RECEPTOR

REFERENCE TO RELATED APPLICATIONS

This application is a continuation-in-part of International Patent Application PCT/GB02/04725 filed October 21, 2002 and published as WO 03,033536 on April 24, 2003, which claims priority from U.S. Provisional Patent Application 60/346,083 filed October 24, 2001 and Great Britain Patent Application 0125183.4 filed October 19, 2001.

Each of the applications and patents mentioned in this document, and each document cited or referenced in each of the above applications and patents, including during the prosecution of each of the applications and patents ("application cited documents") and any manufacturer's instructions or catalogues for any products cited or mentioned in each of the applications and patents and in any of the application cited documents, are hereby incorporated herein by reference. Furthermore, all documents cited in this text, and all documents cited or referenced in documents cited in this text, and any manufacturer's instructions or catalogues for any products cited or mentioned in this text, are hereby incorporated herein by reference.

It is noted that in this disclosure, terms such as " comprises", "comprised", "comprising", "contains", "containing" and the like can have the meaning attributed to them in U.S. Patent law; e.g., they can mean "includes", "included", "including" and the like. Terms such as "consisting essentially of" and "consists essentially of" have the meaning attributed to them in U.S. Patent law, e.g., they allow for the inclusion of additional ingredients or steps that do not detract from the novel or basic characteristics of the invention, i.e., they exclude additional unrecited ingredients or steps that detract from novel or basic characteristics of the invention, and they exclude ingredients or steps of the prior art, such as documents in the art that are cited herein or are incorporated by reference herein, especially as it is a goal of this document to define embodiments that are patentable, e.g., novel, nonobvious, inventive, over the prior art, e.g., over documents cited herein or incorporated by reference herein. And, the terms "consists of" and "consisting

of" have the meaning ascribed to them in U.S. Patent law; namely, that these terms are closed ended.

FIELD

This invention relates to newly identified nucleic acids, polypeptides encoded by them and to their production and use. More particularly, the nucleic acids and polypeptides of the present invention relate to a G-protein coupled receptor (GPCR), hereinafter referred to as "Conrad GPCR", and members of the purinoceptor family of GPCRs. The invention also relates to inhibiting or activating the action of such nucleic acids and polypeptides.

BACKGROUND

It is well established that many medically significant biological processes are mediated by proteins participating in signal transduction pathways that involve G-proteins and/or second messengers, for example, cAMP (Lefkowitz, *Nature*, 1991, 351: 353-354). These proteins are referred to as proteins participating in pathways with G-proteins or "PPG proteins". Some examples of these proteins include the GPC receptors, such as those for adrenergic agents and dopamine (Kobilka, B. K., et al., *Proc. Natl Acad. Sci., USA*, 1987, 84: 46-50; Kobilka B. K., et al., *Science*, 1987, 238: 650-656; Bunzow, J. R., et al., *Nature*, 1988, 336: 783-787), G-proteins themselves, effector proteins, for example, phospholipase C, adenyl cyclase, and phosphodiesterase, and actuator proteins, for example, protein kinase A and protein kinase C (Simon, M. I., et al., *Science*, 1991, 252: 802-8).

For example, in one form of signal transduction, the effect of hormone binding is activation of the enzyme adenylate cyclase inside the cell. Enzyme activation by hormones is dependent on the presence of the nucleotide, GTP. GTP also influences hormone binding. A G-protein connects the hormone receptor to adenylate cyclase. G-protein is shown to exchange GTP for bound GDP when activated by a hormone receptor. The GTP

carrying form then binds to activated adenylate cyclase. Hydrolysis of GTP to GDP, catalysed by the G-protein itself, returns the G-protein to its basal, inactive form. Thus, the G-protein serves a dual role, as an intermediate that relays the signal from receptor to effector, and as a clock that controls the duration of the signal.

The membrane protein gene superfamily of G-protein coupled receptors (GPCRs) has been characterised as having seven putative transmembrane domains. The domains are believed to represent transmembrane α-helices connected by extracellular or cytoplasmic loops. G-protein coupled receptors include a wide range of biologically active receptors, such as hormone, viral, growth factor and neuroreceptors.

G-protein coupled receptors (also known as 7TM receptors) have been characterised as including these seven conserved hydrophobic stretches of about 20 to 30 amino acids, connecting at least eight divergent hydrophilic loops. The G-protein family of coupled receptors includes dopamine receptors which bind to neuroleptic drugs used for treating psychotic and neurological disorders. Other examples of members of this family include, but are not limited to, calcitonin, adrenergic, endothelin, cAMP, adenosine, muscarinic, acetylcholine, serotonin, histamine, thrombin, kinin, follicle stimulating hormone, opsins, endothelial differentiation gene-1, rhodopsins, odorant, and cytomegalovirus receptors.

Most G-protein coupled receptors have single conserved cysteine residues in each of the first two extracellular loops which form disulphide bonds that are believed to stabilise functional protein structure. The 7 transmembrane regions are designated as TM1, TM2, TM3, TM4, TM5, TM6, and TM7. TM3 has been implicated in signal transduction.

Phosphorylation and lipidation (pamitylation or farnesylation) of cysteine residues can influence signal transduction of some G-protein coupled receptors. Most G-protein coupled receptors contain potential phosphorylation sites within the third cytoplasmic loop and/or the carboxy terminus. For several G-protein coupled receptors, such as the β -adrenoreceptor, phosphorylation by protein kinase A and/or specific receptor kinases

mediates receptor desensitization. For some receptors, the ligand binding sites of G-protein coupled receptors are believed to comprise hydrophilic sockets formed by several G-protein coupled receptor transmembrane domains, the sockets being surrounded by hydrophobic residues of the G-protein coupled receptors. The hydrophilic side of each G-protein coupled receptor transmembrane helix is thought to face inward and form a polar ligand binding site. TM3 has been implicated in several G-protein coupled receptors as having a ligand binding site, such as the TM3 aspartate residue. TM5 serines, a TM6 asparagine and TM6 or TM7 phenylalanines or tyrosines are also implicated in ligand binding.

G-protein coupled receptors can be intracellularly coupled by heterotrimeric G-proteins to various intracellular enzymes, ion channels and transporters (see, Johnson et al., *Endoc. Rev.*, 1989, 10: 317-331). Different G-protein α-subunits preferentially stimulate particular effectors to modulate various biological functions in a cell. Phosphorylation of cytoplasmic residues of G-protein coupled receptors has been identified as an important mechanism for the regulation of G-protein coupling of some G-protein coupled receptors. G-protein coupled receptors are found in numerous sites within a mammalian host. Over the past 15 years, nearly 350 therapeutic agents targeting 7 transmembrane (7 TM) receptors have been successfully introduced onto the market.

Thus, G-protein coupled receptors have an established, proven history as therapeutic targets. Clearly there is a need for identification and characterization of further receptors which can play a role in preventing, ameliorating or correcting dysfunctions or diseases, including, but not limited to, infections such as bacterial, fungal, protozoan and viral infections, particularly infections caused by HIV-1 or HIV-2; pain; cancers; diabetes, obesity; anorexia; bulimia; asthma; Parkinson's disease; thrombosis; acute heart failure; hypotension; hypertension; erectile dysfunction; urinary retention; metabolic bone diseases such as osteoporisis and osteo petrosis; angina pectoris; myocardial infarction; ulcers; asthma; allergies; rheumatoid arthritis; inflammatory bowel disease; irritable bowel syndrome benign prostatic hypertrophy; and psychotic and neurological disorders, including anxiety, schizophrenia, manic depression, delirium, dementia, severe mental

retardation and dyskinesias, such as Huntington's disease or Gilles dela Tourett's syndrome.

SUMMARY

According to a first aspect of the present invention, we provide a Conrad GPCR polypeptide comprising the amino acid sequence shown in SEQ ID NO: 3, SEQ ID NO: 5, SEQ ID NO: 9, SEQ ID NO: 11, SEQ ID NO: 14 or SEQ ID NO: 17, or a homologue, variant or derivative thereof.

There is provided, according to a second aspect of the present invention, a nucleic acid capable of encoding a polypeptide according to the first aspect of the invention. Preferably, the nucleic acid comprises the nucleic acid sequence shown in SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 4, SEQ ID NO: 6, SEQ ID NO: 7, SEQ ID NO: 8, SEQ ID NO: 10, SEQ ID NO: 12, SEQ ID NO: 13, SEQ ID NO: 15, SEQ ID NO: 16 or SEQ ID NO: 18, homologue, variant or derivative thereof.

We provide, according to a third aspect of the present invention, a polypeptide comprising a fragment of a polypeptide according to the first aspect of the invention.

Preferably, such a fragment comprises one or more regions which are homologous between a pair of sequences selected from one of SEQ ID NO: 3 and SEQ ID NO: 9, and one of SEQ ID NO: 5 and SEQ ID NO: 11, or which comprises one or more regions which are heterologous between the pair. As a fourth aspect of the present invention, there is provided a nucleic acid capable of encoding a polypeptide according to the third aspect of the invention.

We provide, according to a fifth aspect of the present invention, a vector comprising a nucleic acid according to the second or fourth aspect of the invention.

The present invention, in a sixth aspect, provides a host cell comprising a nucleic acid according to the second or fourth aspect of the invention, or vector according to the fifth aspect of the invention.

In a seventh aspect of the present invention, there is provided a transgenic non-human animal comprising a nucleic acid according to the second or fourth aspect of the invention or a vector according to the fifth aspect of the invention. Preferably, the transgenic non-human animal is a mouse.

According to an eighth aspect of the present invention, we provide use of a polypeptide according to the first or third aspect of the invention in a method of identifying compound which is capable of interacting specifically with a G protein coupled receptor.

We provide, according to a ninth aspect of the invention, use of a transgenic non-human animal according to the seventh aspect of the invention in a method of identifying a compound which is capable of interacting specifically with a G protein coupled receptor.

There is provided, in accordance with a tenth aspect of the present invention, a method for identifying an antagonist of a Conrad GPCR, the method comprising contacting a cell which expresses Conrad receptor with a candidate compound and determining whether the level of cyclic AMP (cAMP) in said cell is lowered as a result of said contacting.

As an eleventh aspect of the invention, we provide a method for identifying a compound capable of lowering the endogenous level of cyclic AMP in a cell which method comprises contacting a cell which expresses a Conrad GPCR with a candidate compound and determining whether the level of cyclic AMP (cAMP) in said cell is lowered as a result of said contacting.

According to a twelfth aspect of the invention, we provide a method for identifying a compound capable of binding to a Conrad GPCR polypeptide, the method comprising contacting a Conrad GPCR polypeptide with a candidate compound and determining whether the candidate compound binds to the Conrad GPCR polypeptide.

We provide, according to a thirteenth aspect of the invention, there is provided a compound identified by a method according to any of the eighth to twelfth aspects of the invention.

According to a fourteenth aspect of the present invention, we provide a compound capable of binding specifically to a polypeptide according to the first or third aspect of the invention.

There is provided, according to a fifteenth aspect of the present invention, use of a polypeptide according to the first or third aspect of the invention, or part thereof; or a nucleic acid according to the second or fourth aspect of the invention, or part thereof, in a method for producing antibodies.

We provide, according to a sixteenth aspect of the present invention, an antibody capable of binding specifically to a polypeptide according to the first or third aspect of the invention, or part thereof; or a polypeptide encoded by a nucleic acid according to the second or fourth aspect of the invention, or part thereof;.

As a seventeenth aspect of the present invention, there is provided a pharmaceutical composition comprising any one or more of the following: a polypeptide according to the first or third aspect of the invention, or part thereof; a polypeptide encoded by a nucleic acid according to the second or fourth aspect of the invention, or part thereof; a vector according to the fifth aspect of the invention; a cell according to the sixth aspect of the invention; a compound according to the thirteenth or fourteenth aspect of the invention; and an antibody according to the sixteenth aspect of the invention, together with a pharmaceutically acceptable carrier or diluent.

We provide, according to a eighteenth aspect of the present invention, a vaccine composition comprising any one or more of the following: a polypeptide according to the first or third aspect of the invention, or part thereof; a polypeptide encoded by a nucleic acid according to the second or fourth aspect of the invention, or part thereof; a vector according to the fifth aspect of the invention; a cell according to the sixth aspect of the invention; a compound according to the thirteenth or fourteenth aspect of the invention; and an antibody according to the sixteenth aspect of the invention.

According to an nineteenth aspect of the present invention, we provide a diagnostic kit for a disease or susceptibility to a disease comprising any one or more of the following: a polypeptide according to the first or third aspect of the invention, or part thereof; a polypeptide encoded by a nucleic acid according to the second or fourth aspect of the invention, or part thereof; a vector according to the fifth aspect of the invention; a cell according to the sixth aspect of the invention; a compound according to the thirteenth or fourteenth aspect of the invention; and an antibody according to the sixteenth aspect of the invention.

We provide, according to a twentieth aspect of the invention, a method of treating a patient suffering from a disease associated with enhanced activity of a Conrad GPCR, which method comprises administering to the patient an antagonist of Conrad GPCR.

There is provided, in accordance with a twenty-first aspect of the present invention, a method of treating a patient suffering from a disease associated with reduced activity of a Conrad GPCR, which method comprises administering to the patient an agonist of Conrad GPCR.

Preferably, the Conrad GPCR comprises a polypeptide having the sequence shown in SEQ ID NO: 3, SEQ ID NO: 5, SEQ ID NO: 9, SEQ ID NO: 11, SEQ ID NO: 14 or SEQ ID NO: 17.

According to a twenty-second aspect of the present invention, we provide a method for treating and/or preventing a disease in a patient, which comprises the step of administering any one or more of the following to the patient: a polypeptide according to the first or third aspect of the invention, or part thereof; a polypeptide encoded by a nucleic acid according to the second or fourth aspect of the invention, or part thereof; a vector according to the fifth aspect of the invention; a cell according to the sixth aspect of the invention; a compound according to the thirteenth or fourteenth aspect of the invention; and an antibody according to the sixteenth aspect of the invention; a pharmaceutical composition according to the seventeenth aspect of the invention; and a vaccine according to the eighteenth aspect of the invention, to the subject.

There is provided, according to a twenty-third aspect of the present invention, an agent comprising a polypeptide according to the first or third aspect of the invention, or part thereof; a polypeptide encoded by a nucleic acid according to the second or fourth aspect of the invention, or part thereof; a vector according to the fifth aspect of the invention; a cell according to the sixth aspect of the invention; a compound according to the thirteenth or fourteenth aspect of the invention; and an antibody according to the sixteenth aspect of the invention, said agent for use in a method of treatment or prophylaxis of disease.

We provide, according to a twenty-fourth aspect of the present invention, use of a polypeptide according to the first or third aspect of the invention, or part thereof; a polypeptide encoded by a nucleic acid according to the second or fourth aspect of the invention, or part thereof; a vector according to the fifth aspect of the invention; a cell according to the sixth aspect of the invention; a compound according to the thirteenth or fourteenth aspect of the invention; and an antibody according to the sixteenth aspect of the invention, for the preparation of a pharmaceutical composition for the treatment or prophylaxis of a disease.

As a twenty-fifth aspect of the present invention, there is provided non-human transgenic animal, characterized in that the transgenic animal comprises an altered Conrad

gene. Preferably, the alteration is selected from the group consisting of: a deletion of Conrad, a mutation in Conrad resulting in loss of function, introduction of an exogenous gene having a nucleotide sequence with targeted or random mutations into Conrad, introduction of an exogenous gene from another species into Conrad, and a combination of any of these.

We provide, according to a twenty-sixth aspect of the present invention, a non-human transgenic animal having a functionally disrupted endogenous Conrad gene, in which the transgenic animal comprises in its genome and expresses a transgene encoding a heterologous Conrad protein.

The present invention, in a twenty-seventh aspect, provides a nucleic acid construct for functionally disrupting a Conrad gene in a host cell, the nucleic acid construct comprising: (a) a non-homologous replacement portion; (b) a first homology region located upstream of the non-homologous replacement portion, the first homology region having a nucleotide sequence with substantial identity to a first Conrad gene sequence; and (c) a second homology region located downstream of the non-homologous replacement portion, the second homology region having a nucleotide sequence with substantial identity to a second Conrad gene sequence, the second Conrad gene sequence having a location downstream of the first Conrad gene sequence in a naturally occurring endogenous Conrad gene.

According to a twenty-eighth aspect of the present invention, we provide a process for producing a Conrad GPCR polypeptide, the method comprising culturing a host cell according to the sixth aspect of the invention under conditions in which a nucleic acid encoding a Conrad GPCR polypeptide is expressed.

There is provided, according to a twenty-ninth aspect of the present invention, a method of detecting the presence of a nucleic acid according to the second or fourth aspect of the invention in a sample, the method comprising contacting the sample with at least

one nucleic acid probe which is specific for said nucleic acid and monitoring said sample for the presence of the nucleic acid.

We provide, according to a thirtieth aspect of the present invention, a method of detecting the presence of a polypeptide according to the first or third aspect of the invention in a sample, the method comprising contacting the sample with an antibody according to the sixteenth aspect of the invention and monitoring said sample for the presence of the polypeptide.

As a thirty-first aspect of the present invention, there is provided a method of diagnosis of a disease or syndrome caused by or associated with increased, decreased or otherwise abnormal expression of Conrad GPCR, the method comprising the steps of: (a) detecting the level or pattern of expression of Conrad GPCR in an animal suffering or suspected to be suffering from such a disease; and (b) comparing the level or pattern of expression with that of a normal animal.

Preferably, the diesase is selected from the group consisting of long QT syndrome-4 with sinus bradycardia disease, mental health wellness-2 disease, psoriasis or susceptibility to psoriasis, dentin dysplasia, type II disease and neutropenia, neonatal alloimmune disease.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1A and 1B are diagrams showing the results of analysis of the human Conrad polypeptide (SEQ ID NO: 3 and SEQ ID NO: 9 respectively) using the HMM structural prediction software of pfam (http://www.sanger.ac.uk/Software/Pfam/search.shtml).

Figure 2 is a diagram showing an expression profile for human Conrad GPCR generated by reverse transcription-polymerase chain reaction (RT-PCR).

Figure 5 is a map of targeting vector pTK5IBLMNL used for constructing transgenic Conrad mice, showing relevant restriction sites.

Figure 3 is a diagram showing the structure of the mouse Conrad locus before knockout.

Figure 4 is a diagram showing the structure of the mouse Conrad locus after knockout.

Sequence Listings

SEQ ID NO: 1 shows a cDNA sequence of human Conrad. **SEQ ID NO: 2** shows an open reading frame derived from SEQ ID NO: 1. **SEQ ID NO: 3** shows an amino acid sequence of human Conrad. **SEQ ID NO: 4** shows an open reading frame of a cDNA for Mouse Conrad, derived from SEQ ID NO: 6. **SEQ ID NO: 5** shows an amino acid sequence of Mouse Conrad, **SEQ ID NO: 6** shows a cDNA sequence of mouse Conrad.

SEQ ID NO: 7 shows a cDNA sequence of human Conrad. **SEQ ID NO: 8** shows an open reading frame derived from SEQ ID NO: 7. **SEQ ID NO: 9** shows an amino acid sequence of human Conrad. **SEQ ID NO: 10** shows an open reading frame of a cDNA for Mouse Conrad, derived from SEQ ID NO: 12. **SEQ ID NO: 11** shows an amino acid sequence of Mouse Conrad, **SEQ ID NO12** shows a cDNA sequence of mouse Conrad.

SEQ ID NO: 13 shows the sequence of a polynucleotide obtained by PCR from SEQ ID NO:7 for expression of a Conrad fusion protein. **SEQ ID NO: 14** shows the amino acid sequence of such a fusion protein. **SEQ ID NO: 15** shows the sequence of a polynucleotide obtained by PCR from SEQ ID NO: 7 for expression of Conrad protein in prokaryotic and eukaryotic cells. **SEQ ID NO: 16** shows the sequence of a polynucleotide obtained by PCR from SEQ ID NO: 7 for expression of a Conrad FLAG fusion protein.

SEQ ID NO:17 shows the amino acid sequence of such a Conrad-FLAG fusion protein.
SEQ ID NO: 18 shows the mouse genomic sequence of Conrad.

DETAILED DESCRIPTION

CONRAD GPCR

Our invention relates in general to a novel G-Protein Coupled Receptor (GPCR), in particular, an orphan purinoceptor type G-protein coupled receptor, which we refer to as Conrad GPCR, as well as homologues, variants or derivatives thereof.

Conrad is structurally related to other proteins of the G-protein coupled receptor family, as shown by the results of sequencing the amplified cDNA products encoding human and mouse Conrad. The cDNA sequence of SEQ ID NO: 1 contains an open reading flame (SEQ ID NO: 2, nucleotide numbers 289 to 1062) encoding a polypeptide of 258 amino acids shown in SEQ ID NO: 3. The cDNA sequence of SEQ ID NO: 7 contains an open reading frame (SEQ ID NO: 8) encoding a polypeptide shown in SEQ ID NO: 9.

Human Conrad is found to map to *Homo sapiens* chromosome 4q26.

Identities and Similarities to Conrad

The amino acid sequence of Conrad has about 38% identity and 59% similarity (using BLAST) in 187 amino acid residues with human neuropeptide NPFF receptor (Accession # Q9Y5X5 : Cikos,S., Gregor,P. and Koppel,J. *Sequence and tissue distribution of a novel G-protein-coupled receptor expressed prominently in human placenta, Biochem. Biophys. Res. Commun.* 256 (2), 352-356 (1999); Elshourbagy,N.A., Ames,R.S., Fitzgerald,L.R., Foley,J.J., Chambers,J.K., Szekeres,P.G., Evans,N.A., Schmidt,D.B. Buckley,P.T., Dytko,G.M., Murdock,P.R., Milligan,G., Groarke,D.A.,Tan,K.B., Shabon,U., Nuthulaganti,P., Wang,D.Y., Wilson,S., Bergsma,D.J. and Sarau,H.M. *Receptor for the pain modulatory neuropeptides FF and AF is an orphan*

G protein-coupled receptor. J. Biol. Chem. 275 (34), 25965-25971 (2000); Bonini,J.A., Jones,K.A., Adham,N., Forray,C., Artymyshyn,R., Durkin,M.M., Smith,K.E., Tamm,J.A., Boteju,L.W., Lakhlani,P.P., Raddatz,R., Yao,W.-J., Ogozalek,K.L., Boyle,N., Kouranova,E.V., Quan,Y., Vaysse,P.J., Wetzel,J.M., Branchek,T.A., Gerald,C. and Borowsky,B. *Identification and characterization of two G protein-coupled receptors for neuropeptide FF. J. Biol. Chem.* 275 (50), 39324-39331 (2000)).

The nucleotide sequence of Conrad (SEQ ID NO:1) has 100% identity (using BLAST) in 432 nucleotide residues with the anonymous Homo sapiens EST clear cell tumour cDNA from (Accession # AI308124 NCI-CGAP http://www.ncbi.nlm.nih.gov/ncicgap 08-APR-1999) and with an anonymous *Homo sapiens* EST clear cell tumour cDNA from (Accession #AI307658 NCI-CGAP http://www.ncbi.nlm.nih.gov/ncicgap 08-APR-1999). Furthermore, Conrad (SEQ ID NO: 1) is about 98% identical in 730 nucleotide residues to the anonymous Homo sapiens EST hypernephroma, cell line cDNA clone (Accession # BG169612 NCI-CGAP http://www.ncbi.nlm.nih.gov/ncicgap, 07-FEB-1998).

Analysis of the Conrad polypeptide (SEQ ID NO: 3 and SEQ ID NO: 9) using the HMM structural prediction software of pfam (http://www.sanger.ac.uk/Software/Pfam/search.shtml) confirms that Conrad peptide is a GPCR of the 7TM-1 structural class (see Figure 1A and Figure 1B).

The mouse orthologue of the human Conrad GPCR has been cloned, and its nucleic acid sequences are shown as SEQ ID NO: 4 and SEQ ID NO:10 and the amino acid sequences are shown as SEQ ID NO: 5 and SEQ ID NO: 11 respectively. The mouse Conrad cDNA sequence of SEQ ID NO: 6 contains an open reading frame shown in SEQ ID NO: 4. The mouse Conrad cDNA sequence of SEQ ID NO: 12 contains an open reading frame shown in SEQ ID NO: 10.

The mouse Conrad cDNAs SEQ ID NOs: 4 and 10 show a high degree of identity with the human Conrad GPCR sequences (SEQ ID NOs: 2 and 8), while the amino acid

sequences (SEQ ID Nos: 5 and 11) of mouse Conrad GPCR show a high degree of identity and similarity with human Conrad GPCR (SEQ ID NOs: 3 and 9). Murine Conrad (SEQ ID NO: 5) is 86.8% identical and 90.7% similar to human Conrad (SEQ ID NO: 3). Murine Conrad (SEQ ID NO:11) is 83% identical and 89% similar to human Conrad (SEQ ID NO: 9).

Human and mouse Conrad GPCR are therefore members of a large family of G Protein Coupled Receptors (GPCRs).

Expression Profile of Conrad

Polymerase chain reaction (PCR) amplification of Conrad cDNA detects expression of Conrad to varying abundance in human heart, brain, lung and testis. An expression profile of Conrad GPCR is shown in Figure 2. Using Conrad cDNA of SEQ ID NO: 1 to search the human EST data sources by BLASTN, identities are found in cDNA derived from libraries originating from Human hypernephroma cell line from kidney (Accession # BG169612), tumour (clear cell type) from kidney (Accession # AI307658 and AI308124); Mouse retina (accession # BB277215 and BB642180) and mouse diencephalon (accession # BB626475 and BB084541).

This indicates that Conrad is expressed in these normal or abnormal tissues. Accordingly, the Conrad polypeptides, nucleic acids, probes, antibodies, expression vectors and ligands are useful for detection, diagnosis, treatment and other assays for diseases associated with over-, under- and abnormal expression of Conrad GPCR in these and other tissues.

This and other embodiments of the invention will be described in further detail below.

METHODS EMPLOYED

The practice of the present invention will employ, unless otherwise indicated, conventional techniques of chemistry, molecular biology, microbiology, recombinant DNA and immunology, which are within the capabilities of a person of ordinary skill in the art. Such techniques are explained in the literature. See, for example, J. Sambrook, E. F. Fritsch, and T. Maniatis, 1989, *Molecular Cloning: A Laboratory Manual*, Second Edition, Books 1-3, Cold Spring Harbor Laboratory Press; Ausubel, F. M. et al. (1995 and periodic supplements; *Current Protocols in Molecular Biology*, ch. 9, 13, and 16, John Wiley & Sons, New York, N.Y.); B. Roe, J. Crabtree, and A. Kahn, 1996, *DNA Isolation and Sequencing: Essential Techniques*, John Wiley & Sons; J. M. Polak and James O'D. McGee, 1990, *In Situ Hybridization: Principles and Practice*; Oxford University Press; M. J. Gait (Editor), 1984, *Oligonucleotide Synthesis: A Practical Approach*, Irl Press; D. M. J. Lilley and J. E. Dahlberg, 1992, *Methods of Enzymology: DNA Structure Part A: Synthesis and Physical Analysis of DNA* Methods in Enzymology, Academic Press; Using Antibodies : A Laboratory Manual : Portable Protocol NO. I by Edward Harlow, David Lane, Ed Harlow (1999, Cold Spring Harbor Laboratory Press, ISBN 0-87969-544-7); Antibodies : A Laboratory Manual by Ed Harlow (Editor), David Lane (Editor) (1988, Cold Spring Harbor Laboratory Press, ISBN 0-87969-314-2), 1855. Handbook of Drug Screening, edited by Ramakrishna Seethala, Prabhavathi B. Fernandes (2001, New York, NY, Marcel Dekker, ISBN 0-8247-0562-9); and Lab Ref: A Handbook of Recipes, Reagents, and Other Reference Tools for Use at the Bench, Edited Jane Roskams and Linda Rodgers, 2002, Cold Spring Harbor Laboratory, ISBN 0-87969-630-3. Each of these general texts is herein incorporated by reference.

CONRAD GPCR POLYPEPTIDES

As used here, the term "Conrad GPCR polypeptide" is intended to refer to a polypeptide comprising the amino acid sequence shown in SEQ ID NO: 3, SEQ ID NO: 5, SEQ ID NO: 9, SEQ ID NO: 11, SEQ ID NO: 14 or SEQ ID NO: 17, or a homologue,

variant or derivative thereof. Preferably, the polypeptide comprises or is a homologue, variant or derivative of the sequence shown in SEQ ID NO: 3, or in SEQ ID NO: 9.

"Polypeptide" refers to any peptide or protein comprising two or more amino acids joined to each other by peptide bonds or modified peptide bonds, i.e., peptide isosteres. "Polypeptide" refers to both short chains, commonly referred to as peptides, oligopeptides or oligomers, and to longer chains, generally referred to as proteins. Polypeptides may contain amino acids other than the 20 gene-encoded amino acids.

"Polypeptides" include amino acid sequences modified either by natural processes, such as post-translational processing, or by chemical modification techniques which are well known in the art. Such modifications are well described in basic texts and in more detailed monographs, as well as in a voluminous research literature. Modifications can occur anywhere in a polypeptide, including the peptide backbone, the amino acid side-chains and the amino or carboxyl termini. It will be appreciated that the same type of modification may be present in the same or varying degrees at several sites in a given polypeptide. Also, a given polypeptide may contain many types of modifications.

Polypeptides may be branched as a result of ubiquitination, and they may be cyclic, with or without branching. Cyclic, branched and branched cyclic polypeptides may result from posttranslation natural processes or may be made by synthetic methods. Modifications include acetylation, acylation, ADP-ribosylation, amidation, covalent attachment of flavin, covalent attachment of a heme moiety, covalent attachment of a nucleotide or nucleotide derivative, covalent attachment of a lipid or lipid derivative, covalent attachment of phosphotidylinositol, cross-inking, cyclization, disulfide bond formation, demethylation, formation of covalent cross-inks, formation of cystine, formation of pyroglutamate, formylation, gamma-carboxylation, glycosylation, GPI anchor formation, hydroxylation, iodination, methylation, myristoylation, oxidation, proteolytic processing, phosphorylation, prenylation, racemization, selenoylation, sulfation, transfer-RNA mediated addition of amino acids to proteins such as arginylation, and ubiquitination. See, for instance, *Proteins - Structure and Molecular Properties*, 2nd

Ed., T. E. Creighton, W. H. Freeman and Company, New York, 1993 and Wold, F., *Posttranslational Protein Modifications: Perspectives and Prospects*, pgs. 1-12 in *Posttranslational Covalent Modification of Proteins*, B. C. Johnson, Ed., Academic Press, New York, 1983; Seifter et al., "Analysis for protein modifications and nonprotein cofactors", *Meth Enzymol* (1990) 182:626-646 and Rattan et aL, "Protein Synthesis: Posttranslational Modifications and Aging", *Ann NY Acad Sci* (1992) 663:48-62.

The terms "variant", "homologue", "derivative" or "fragment" as used in this document include any substitution of, variation of, modification of, replacement of, deletion of or addition of one (or more) amino acid from or to a sequence. Unless the context admits otherwise, references to "Conrad" and "Conrad GPCR" include references to such variants, homologues, derivatives and fragments of Conrad.

Preferably, as applied to Conrad, the resultant amino acid sequence has GPCR activity, more preferably having at least the same activity of the Conrad GPCR shown as SEQ ID NO: 3, SEQ ID NO: 5, SEQ ID NO: 9, SEQ ID NO: 11, SEQ ID NO: 14 or SEQ ID NO: 17. In particular, the term "homologue" covers identity with respect to structure and/or function providing the resultant amino acid sequence has GPCR activity. With respect to sequence identity (i.e. similarity), preferably there is at least 70%, more preferably at least 75%, more preferably at least 85%, even more preferably at least 90% sequence identity. More preferably there is at least 95%, more preferably at least 98%, sequence identity. These terms also encompass polypeptides derived from amino acids which are allelic variations of the Conrad GPCR nucleic acid sequence.

Where reference is made to the "receptor activity" or "biological activity" of a receptor such as Conrad GPCR, these terms are intended to refer to the metabolic or physiological function of the Conrad receptor, including similar activities or improved activities or these activities with decreased undesirable side effects. Also included are antigenic and immunogenic activities of the Conrad receptor. Examples of GPCR activity, and methods of assaying and quantifying these activities, are known in the art, and are described in detail elsewhere in this document.

As used herein a "deletion" is defined as a change in either nucleotide or amino acid sequence in which one or more nucleotides or amino acid residues, respectively, are absent. As used herein an "insertion" or "addition" is that change in a nucleotide or amino acid sequence which has resulted in the addition of one or more nucleotides or amino acid residues, respectively, as compared to the naturally occurring substance. As used herein "substitution" results from the replacement of one or more nucleotides or amino acids by different nucleotides or amino acids, respectively.

Conrad polypeptides as described here may also have deletions, insertions or substitutions of amino acid residues which produce a silent change and result in a functionally equivalent amino acid sequence. Deliberate amino acid substitutions may be made on the basis of similarity in polarity, charge, solubility, hydrophobicity, hydrophilicity, and/or the amphipathic nature of the residues. For example, negatively charged amino acids include aspartic acid and glutamic acid; positively charged amino acids include lysine and arginine; and amino acids with uncharged polar head groups having similar hydrophilicity values include leucine, isoleucine, valine, glycine, alanine, asparagine, glutamine, serine, threonine, phenylalanine, and tyrosine.

Conservative substitutions may be made, for example according to the table below. Amino acids in the same block in the second column and preferably in the same line in the third column may be substituted for each other:

ALIPHATIC	Non-polar	G A P
		I L V
	Polar - uncharged	C S T M
		N Q
	Polar - charged	D E
		K R
AROMATIC		H F W Y

Conrad polypeptides may further comprise heterologous amino acid sequences, typically at the N-terminus or C-terminus, preferably the N-terminus. Heterologous sequences may include sequences that affect intra or extracellular protein targeting (such as leader sequences). Heterologous sequences may also include sequences that increase

the immunogenicity of the polypeptide and/or which facilitate identification, extraction and/or purification of the polypeptides. Another heterologous sequence that is particularly preferred is a polyamino acid sequence such as polyhistidine which is preferably N-terminal. A polyhistidine sequence of at least 10 amino acids, preferably at least 17 amino acids but fewer than 50 amino acids is especially preferred.

The Conrad GPCR polypeptides may be in the form of the "mature" protein or may be a part of a larger protein such as a fusion protein. It is often advantageous to include an additional amino acid sequence which contains secretory or leader sequences, pro-sequences, sequences which aid in purification such as multiple histidine residues, or an additional sequence for stability during recombinant production.

Conrad polypeptides are advantageously made by recombinant means, using known techniques. However they may also be made by synthetic means using techniques well known to skilled persons such as solid phase synthesis. Such polypeptides may also be produced as fusion proteins, for example to aid in extraction and purification. Examples of fusion protein partners include glutathione-S-transferase (GST), 6xHis, GAL4 (DNA binding and/or transcriptional activation domains) and β-galactosidase. It may also be convenient to include a proteolytic cleavage site between the fusion protein partner and the protein sequence of interest to allow removal of fusion protein sequences, such as a thrombin cleavage site. Preferably the fusion protein will not hinder the function of the protein of interest sequence.

Conrad polypeptides may be in a substantially isolated form. This term is intended to refer to alteration by the hand of man from the natural state. If an "isolated" composition or substance occurs in nature, it has been changed or removed from its original environment, or both. For example, a polynucleotide, nucleic acid or a polypeptide naturally present in a living animal is not "isolated," but the same polynucleotide, nucleic acid or polypeptide separated from the coexisting materials of its natural state is "isolated", as the term is employed herein.

It will however be understood that the Conrad GPCR protein may be mixed with carriers or diluents which will not interfere with the intended purpose of the protein and still be regarded as substantially isolated. A polypeptide as described here may also be in a substantially purified form, in which case it will generally comprise the protein in a preparation in which more than 90%, for example, 95%, 98% or 99% of the protein in the preparation is a Conrad GPCR polypeptide.

We also describe peptides comprising a portion of a Conrad polypeptide. Thus, fragments of Conrad GPCR and its homologues, variants or derivatives are included. The peptides of described here may be between 2 and 200 amino acids, preferably between 4 and 40 amino acids in length. The peptide may be derived from a Conrad GPCR polypeptide as disclosed here, for example by digestion with a suitable enzyme, such as trypsin. Alternatively the peptide, fragment, etc may be made by recombinant means, or synthesised synthetically,

The term "peptide" includes the various synthetic peptide variations known in the art, such as a retroinverso D peptides. The peptide may be an antigenic determinant and/or a T-cell epitope. The peptide may be immunogenic *in vivo*. Preferably the peptide is capable of inducing neutralising antibodies *in vivo*.

By aligning Conrad GPCR sequences from different species, it is possible to determine which regions of the amino acid sequence are conserved between different species ("homologous regions"), and which regions vary between the different species ("heterologous regions").

The Conrad polypeptides as described here may therefore comprise a sequence which corresponds to at least part of a homologous region. A homologous region shows a high degree of homology between at least two species. For example, the homologous region may show at least 70%, preferably at least 80%, more preferably at least 90%, even more preferably at least 95% identity at the amino acid level using the tests described above. Peptides which comprise a sequence which corresponds to a homologous region

may be used in therapeutic strategies as explained in further detail below. Alternatively, the Conrad GPCR peptide may comprise a sequence which corresponds to at least part of a heterologous region. A heterologous region shows a low degree of homology between at least two species.

CONRAD GPCR POLYNUCLEOTIDES AND NUCLEIC ACIDS

Conrad polynucleotides, Conrad nucleotides and Conrad nucleic acids, methods of production, uses of these, etc, as described in further detail elsewhere in this document are disclosed.

The terms "Conrad polynucleotide", "Conrad nucleotide" and "Conrad nucleic acid" may be used interchangeably, and are intended to refer to a polynucleotide/nucleic acid comprising a nucleic acid sequence as shown in SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 4, SEQ ID NO: 6, SEQ ID NO: 7, SEQ ID NO: 8, SEQ ID NO: 10, SEQ ID NO: 12, SEQ ID NO: 13, SEQ ID NO: 15, SEQ ID NO: 16 or SEQ ID NO: 18, or a homologue, variant or derivative thereof. Preferably, the polynucleotide/nucleic acid comprises or is a homologue, variant or derivative of the nucleic acid sequence SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 7 or SEQ ID NO: 8, most preferably, SEQ ID NO: 2 and SEQ ID NO: 8.

These terms are also intended to include a nucleic acid sequence capable of encoding a polypeptides and/or a peptide as described here, i.e., a Conrad polypeptide. Thus, Conrad GPCR polynucleotides and nucleic acids comprise a nucleotide sequence capable of encoding a polypeptide comprising the amino acid sequence shown in SEQ ID NO: 3, SEQ ID NO: 5, SEQ ID NO: 9, SEQ ID NO: 11, SEQ ID NO: 14 or SEQ ID NO: 17, or a homologue, variant or derivative thereof. Preferably, the Conrad GPCR polynucleotides and nucleic acids comprise a nucleotide sequence capable of encoding a polypeptide comprising the amino acid sequence shown in SEQ ID NO: 3, SEQ ID NO: 9, SEQ ID NO: 5 or SEQ ID NO: 11, or a homologue, variant or derivative thereof.

"Polynucleotide" generally refers to any polyribonucleotide or polydeoxribonucleotide, which may be unmodified RNA or DNA or modified RNA or DNA. "Polynucleotides" include, without limitation single- and double-stranded DNA, DNA that is a mixture of single- and double-stranded regions, single- and double-stranded RNA, and RNA that is mixture of single- and double-stranded regions, hybrid molecules comprising DNA and RNA that may be single-stranded or, more typically, double-stranded or a mixture of single- and double-stranded regions. In addition, "polynucleotide" refers to triple-stranded regions comprising RNA or DNA or both RNA and DNA. The term polynucleotide also includes DNAs or RNAs containing one or more modified bases and DNAs or RNAs with backbones modified for stability or for other reasons. "Modified" bases include, for example, tritylated bases and unusual bases such as inosine. A variety of modifications has been made to DNA and RNA; thus, "polynucleotide" embraces chemically, enzymatically or metabolically modified forms of polynucleotides as typically found in nature, as well as the chemical forms of DNA and RNA characteristic of viruses and cells. "Polynucleotide" also embraces relatively short polynucleotides, often referred to as oligonucleotides.

It will be understood by the skilled person that numerous nucleotide sequences can encode the same polypeptide as a result of the degeneracy of the genetic code.

As used herein, the term "nucleotide sequence" refers to nucleotide sequences, oligonucleotide sequences, polynucleotide sequences and variants, homologues, fragments and derivatives thereof (such as portions thereof). The nucleotide sequence may be DNA or RNA of genomic or synthetic or recombinant origin which may be double-stranded or single-stranded whether representing the sense or antisense strand or combinations thereof. The term nucleotide sequence may be prepared by use of recombinant DNA techniques (for example, recombinant DNA).

Preferably, the term "nucleotide sequence" means DNA.

The terms "variant", "homologue", "derivative" or "fragment" as used in this document include any substitution of, variation of, modification of, replacement of, deletion of or addition of one (or more) nucleic acids from or to the sequence of a Conrad nucleotide sequence. Unless the context admits otherwise, references to "Conrad" and "Conrad GPCR" include references to such variants, homologues, derivatives and fragments of Conrad.

Preferably, the resultant nucleotide sequence encodes a polypeptide having GPCR activity, preferably having at least the same activity of the GPCR shown as SEQ ID NO: 3, SEQ ID NO: 5, SEQ ID NO: 9 or SEQ ID NO: 11. Preferably, the term "homologue" is intended to cover identity with respect to structure and/or function such that the resultant nucleotide sequence encodes a polypeptide which has GPCR activity. With respect to sequence identity (i.e. similarity), preferably there is at least 70%, more preferably at least 75%, more preferably at least 85%, more preferably at least 90% sequence identity. More preferably there is at least 95%, more preferably at least 98%, sequence identity. These terms also encompass allelic variations of the sequences.

CONRAD GPCR ASSOCIATED DISEASES

According to the methods and compositions described here, Conrad GPCR is useful for treating and diagnosing a range of diseases.

We demonstrate here that human Conrad maps to *Homo sapiens* chromosome 4q26. Accordingly, in a specific embodiment, Conrad GPCR may be used to treat or diagnose a disease which maps to this locus, chromosomal band, region, arm or the same chromosome.

Known diseases which have been determined as being linked to the same locus, chromosomal band, region, arm or chromosome as the chromosomal location of Conrad GPCR (i.e., chromosome 4q26) include the following (locations in brackets): Long QT syndrome-4 with sinus bradycardia disease (LQT4; gene map locus 4q25-q27); mental

health wellness-2 disease: MHW2 (Gene map locus 4q); Susceptibility to psoriasis, PSORIASIS SUSCEPTIBILITY 3 (PSORS3; Gene map locus 4q); dentin dysplasia, type II disease: DTDP2 (Gene map locus 4q); and neutropenia, neonatal alloimmune disease: LAG5 (Gene map chromosome 4).

Accordingly, according to a preferred embodiment, Conrad GPCR may be used to diagnose or treat, by any means as described in this document, neutropenia, neonatal alloimmune disease. More preferably, Conrad GPCR is used to diagnose or treat mental health wellness-2 disease, psoriasis or susceptibility to psoriasis, or dentin dysplasia, type II disease. Most preferably, Conrad GPCR is used to diagnose or treat Long QT syndrome-4 with sinus bradycardia disease. As noted above, Conrad GPCR may be used to diagnose and/or treat any of these specific diseases using any of the methods and compositions described here.

In particular, we specifically envisage the use of nucleic acids, vectors comprising Conrad GPCR nucleic acids, polypeptides, including homologues, variants or derivatives thereof, pharmaceutical compositions, host cells, and transgenic animals comprising Conrad GPCR nucleic acids and/or polypeptides, for the treatment or diagnosis of the specific diseases listed above. Furthermore, we envisage the use of compounds capable of interacting with or binding to Conrad GPCR, preferably antagonists of a Conrad GPCR, preferably a compound capable of lowering the endogenous level of cyclic AMP in a cell, antibodies against Conrad GPCR, as well as methods of making or identifying these, in diagnosis or treatment of the specific diseases mentioned above. In particular, we include the use of any of these compounds, compositions, molecules, etc, in the production of vaccines for treatment or prevention of the specific diseases. We also disclose diagnostic kits for the detection of the specific diseases in an individual.

Methods of linkage mapping to identify such or further specific diseases treatable or diagnosable by use of Conrad GPCR are known in the art, and are also described elsewhere in this document.

CALCULATION OF SEQUENCE HOMOLOGY

Sequence identity with respect to any of the sequences presented here can be determined by a simple "eyeball" comparison (i.e. a strict comparison) of any one or more of the sequences with another sequence to see if that other sequence has, for example, at least 70% sequence identity to the sequence(s).

Relative sequence identity can also be determined by commercially available computer programs that can calculate % identity between two or more sequences using any suitable algorithm for determining identity, using for example default parameters. A typical example of such a computer program is CLUSTAL. Other computer program methods to determine identify and similarity between the two sequences include but are not limited to the GCG program package (Devereux *et al* 1984 Nucleic Acids Research 12: 387) and FASTA (Atschul *et al* 1990 J Molec Biol 403-410).

% homology may be calculated over contiguous sequences, i.e. one sequence is aligned with the other sequence and each amino acid in one sequence is directly compared with the corresponding amino acid in the other sequence, one residue at a time. This is called an "ungapped" alignment. Typically, such ungapped alignments are performed only over a relatively short number of residues.

Although this is a very simple and consistent method, it fails to take into consideration that, for example, in an otherwise identical pair of sequences, one insertion or deletion will cause the following amino acid residues to be put out of alignment, thus potentially resulting in a large reduction in % homology when a global alignment is performed. Consequently, most sequence comparison methods are designed to produce optimal alignments that take into consideration possible insertions and deletions without penalising unduly the overall homology score. This is achieved by inserting "gaps" in the sequence alignment to try to maximise local homology.

However, these more complex methods assign "gap penalties" to each gap that occurs in the alignment so that, for the same number of identical amino acids, a sequence alignment with as few gaps as possible - reflecting higher relatedness between the two compared sequences - will achieve a higher score than one with many gaps. "Affine gap costs" are typically used that charge a relatively high cost for the existence of a gap and a smaller penalty for each subsequent residue in the gap. This is the most commonly used gap scoring system. High gap penalties will of course produce optimised alignments with fewer gaps. Most alignment programs allow the gap penalties to be modified. However, it is preferred to use the default values when using such software for sequence comparisons. For example, when using the GCG Wisconsin Bestfit package the default gap penalty for amino acid sequences is -12 for a gap and -4 for each extension.

Calculation of maximum % homology therefore firstly requires the production of an optimal alignment, taking into consideration gap penalties. A suitable computer program for carrying out such an alignment is the GCG Wisconsin Bestfit package (University of Wisconsin, U.S.A.; Devereux *et al.,* 1984, Nucleic Acids Research 12:387). Examples of other software than can perform sequence comparisons include, but are not limited to, the BLAST package (Ausubel *et al.*, 1999 *ibid* – Chapter 18), FASTA (Atschul *et al.,* 1990, J. Mol. Biol., 403-410) and the GENEWORKS suite of comparison tools. Both BLAST and FASTA are available for offline and online searching (Ausubel *et al.*, 1999 *ibid*, pages 7-58 to 7-60).

Although the final % homology can be measured in terms of identity, the alignment process itself is typically not based on an all-or-nothing pair comparison. Instead, a scaled similarity score matrix is generally used that assigns scores to each pairwise comparison based on chemical similarity or evolutionary distance. An example of such a matrix commonly used is the BLOSUM62 matrix - the default matrix for the BLAST suite of programs. GCG Wisconsin programs generally use either the public default values or a custom symbol comparison table if supplied. It is preferred to use the public default values for the GCG package, or in the case of other software, the default matrix, such as BLOSUM62.

Advantageously, the BLAST algorithm is employed, with parameters set to default values. The BLAST algorithm is described in detail at http://www.ncbi.nih.gov/BLAST/blast_help.html, which is incorporated herein by reference. The search parameters are defined as follows, can be advantageously set to the defined default parameters.

Advantageously, "substantial identity" when assessed by BLAST equates to sequences which match with an EXPECT value of at least about 7, preferably at least about 9 and most preferably 10 or more. The default threshold for EXPECT in BLAST searching is usually 10.

BLAST (Basic Local Alignment Search Tool) is the heuristic search algorithm employed by the programs blastp, blastn, blastx, tblastn, and tblastx; these programs ascribe significance to their findings using the statistical methods of Karlin and Altschul (Karlin and Altschul 1990, *Proc. Natl. Acad. Sci. USA* 87:2264-68; Karlin and Altschul, 1993, *Proc. Natl. Acad. Sci. USA* 90:5873-7; see http://www.ncbi.nih.gov/BLAST/blast_help.html) with a few enhancements. The BLAST programs are tailored for sequence similarity searching, for example to identify homologues to a query sequence. For a discussion of basic issues in similarity searching of sequence databases, see Altschul *et al* (1994) Nature Genetics 6:119-129.

The five BLAST programs available at http://www.ncbi.nlm.nih.gov perform the following tasks: **blastp** - compares an amino acid query sequence against a protein sequence database; **blastn** - compares a nucleotide query sequence against a nucleotide sequence database; **blastx** - compares the six-frame conceptual translation products of a nucleotide query sequence (both strands) against a protein sequence database; **tblastn** - compares a protein query sequence against a nucleotide sequence database dynamically translated in all six reading frames (both strands); **tblastx** - compares the six-frame translations of a nucleotide query sequence against the six-frame translations of a nucleotide sequence database.

BLAST uses the following search parameters:

HISTOGRAM - Display a histogram of scores for each search; default is yes. (See parameter H in the BLAST Manual).

DESCRIPTIONS - Restricts the number of short descriptions of matching sequences reported to the number specified; default limit is 100 descriptions. (See parameter V in the manual page).

EXPECT - The statistical significance threshold for reporting matches against database sequences; the default value is 10, such that 10 matches are expected to be found merely by chance, according to the stochastic model of Karlin and Altschul (1990). If the statistical significance ascribed to a match is greater than the EXPECT threshold, the match will not be reported. Lower EXPECT thresholds are more stringent, leading to fewer chance matches being reported. Fractional values are acceptable. (See parameter E in the BLAST Manual).

CUTOFF - Cutoff score for reporting high-scoring segment pairs. The default value is calculated from the EXPECT value (see above). HSPs are reported for a database sequence only if the statistical significance ascribed to them is at least as high as would be ascribed to a lone HSP having a score equal to the CUTOFF value. Higher CUTOFF values are more stringent, leading to fewer chance matches being reported. (See parameter S in the BLAST Manual). Typically, significance thresholds can be more intuitively managed using EXPECT.

ALIGNMENTS - Restricts database sequences to the number specified for which high-scoring segment pairs (HSPs) are reported; the default limit is 50. If more database sequences than this happen to satisfy the statistical significance threshold for reporting (see EXPECT and CUTOFF below), only the matches ascribed the greatest statistical significance are reported. (See parameter B in the BLAST Manual).

MATRIX - Specify an alternate scoring matrix for BLASTP, BLASTX, TBLASTN and TBLASTX. The default matrix is BLOSUM62 (Henikoff & Henikoff, 1992). The valid alternative choices include: PAM40, PAM120, PAM250 and IDENTITY. No alternate scoring matrices are available for BLASTN; specifying the MATRIX directive in BLASTN requests returns an error response.

STRAND - Restrict a TBLASTN search to just the top or bottom strand of the database sequences; or restrict a BLASTN, BLASTX or TBLASTX search to just reading frames on the top or bottom strand of the query sequence.

FILTER - Mask off segments of the query sequence that have low compositional complexity, as determined by the SEG program of Wootton & Federhen (1993) Computers and Chemistry 17:149-163, or segments consisting of short-periodicity internal repeats, as determined by the XNU program of Claverie & States (1993) Computers and Chemistry 17:191-201, or, for BLASTN, by the DUST program of Tatusov and Lipman (see http://www.ncbi.nlm.nih.gov). Filtering can eliminate statistically significant but biologically uninteresting reports from the blast output (e.g., hits against common acidic-, basic- or proline-rich regions), leaving the more biologically interesting regions of the query sequence available for specific matching against database sequences.

Low complexity sequence found by a filter program is substituted using the letter "N" in nucleotide sequence (e.g., "NNNNNNNNNNNNN") and the letter "X" in protein sequences (e.g., "XXXXXXXXX").

Filtering is only applied to the query sequence (or its translation products), not to database sequences. Default filtering is DUST for BLASTN, SEG for other programs.

It is not unusual for nothing at all to be masked by SEG, XNU, or both, when applied to sequences in SWISS-PROT, so filtering should not be expected to always yield an effect. Furthermore, in some cases, sequences are masked in their entirety, indicating

that the statistical significance of any matches reported against the unfiltered query sequence should be suspect.

NCBI-gi - Causes NCBI gi identifiers to be shown in the output, in addition to the accession and/or locus name.

Most preferably, sequence comparisons are conducted using the simple BLAST search algorithm provided at http://www.ncbi.nlm.nih.gov/BLAST. In some embodiments, no gap penalties are used when determining sequence identity.

HYBRIDISATION

We also describe nucleotide sequences that are capable of hybridising to the sequences presented herein, or any fragment or derivative thereof, or to the complement of any of the above.

Hybridization means a "process by which a strand of nucleic acid joins with a complementary strand through base pairing" (Coombs J (1994) Dictionary of Biotechnology, Stockton Press, New York NY) as well as the process of amplification as carried out in polymerase chain reaction technologies as described in Dieffenbach CW and GS Dveksler (1995, PCR Primer, a Laboratory Manual, Cold Spring Harbor Press, Plainview NY).

Hybridization conditions are based on the melting temperature (Tm) of the nucleic acid binding complex, as taught in Berger and Kimmel (1987, Guide to Molecular Cloning Techniques, Methods in Enzymology, Vol 152, Academic Press, San Diego CA), and confer a defined "stringency" as explained below.

Nucleotide sequences capable of selectively hybridising to the nucleotide sequences presented herein, or to their complement, will be generally at least 70%, preferably at least 75%, more preferably at least 85 or 90% and even more preferably at least 95% or 98%

homologous to the corresponding nucleotide sequences presented herein over a region of at least 20, preferably at least 25 or 30, for instance at least 40, 60 or 100 or more contiguous nucleotides. Preferred nucleotide sequences will comprise regions homologous to SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 4, SEQ ID NO: 6, SEQ ID NO: 7, SEQ ID NO: 8, SEQ ID NO: 10, SEQ ID NO: 12, preferably at least 70%, 80% or 90% and more preferably at least 95% homologous to one of the sequences.

The term "selectively hybridizable" means that the nucleotide sequence used as a probe is used under conditions where a target nucleotide sequence is found to hybridize to the probe at a level significantly above background. The background hybridization may occur because of other nucleotide sequences present, for example, in the cDNA or genomic DNA library being screened. In this event, background implies a level of signal generated by interaction between the probe and a non-specific DNA member of the library which is less than 10 fold, preferably less than 100 fold as intense as the specific interaction observed with the target DNA. The intensity of interaction may be measured, for example, by radiolabelling the probe, e.g. with ^{32}P.

Also included are nucleotide sequences that are capable of hybridizing to the nucleotide sequences presented herein under conditions of intermediate to maximal stringency. Hybridization conditions are based on the melting temperature (Tm) of the nucleic acid binding complex, as taught in Berger and Kimmel (1987, Guide to Molecular Cloning Techniques, Methods in Enzymology, Vol 152, Academic Press, San Diego CA), and confer a defined "stringency" as explained below.

Maximum stringency typically occurs at about Tm-5°C (5°C below the Tm of the probe); high stringency at about 5°C to 10°C below Tm; intermediate stringency at about 10°C to 20°C below Tm; and low stringency at about 20°C to 25°C below Tm. As will be understood by those of skill in the art, a maximum stringency hybridization can be used to identify or detect identical nucleotide sequences while an intermediate (or low) stringency hybridization can be used to identify or detect similar or related nucleotide sequences.

In a preferred embodiment, nucleotide sequences are disclosed that can hybridise to one or more of the Conrad GPCR nucleotide sequences as described here under stringent conditions (e.g. 65°C and 0.1xSSC {1xSSC = 0.15 M NaCl, 0.015 M Na_3 Citrate pH 7.0). Where the nucleotide sequence is double-stranded, both strands of the duplex, either individually or in combination, are encompassed. Where the nucleotide sequence is single-stranded, it is to be understood that the complementary sequence of that nucleotide sequence is also included.

We further describe nucleotide sequences that are capable of hybridising to the sequences that are complementary to the sequences presented herein, or any fragment or derivative thereof. Likewise, we provide nucleotide sequences that are complementary to sequences that are capable of hybridising to such sequences. These types of nucleotide sequences are examples of variant nucleotide sequences. In this respect, the term "variant" encompasses sequences that are complementary to sequences that are capable of hydridising to the nucleotide sequences presented herein. Preferably, however, the term "variant" encompasses sequences that are complementary to sequences that are capable of hydridising under stringent conditions (eg. 65°C and 0.1xSSC {1xSSC = 0.15 M NaCl, 0.015 Na_3 citrate pH 7.0}) to the nucleotide sequences presented herein.

CLONING OF CONRAD GPCR AND HOMOLOGUES

We describe nucleotide sequences that are complementary to the sequences presented here, or any fragment or derivative thereof. If the sequence is complementary to a fragment thereof then that sequence can be used as a probe to identify and clone similar GPCR sequences in other organisms etc.

This document thus enables the cloning of Conrad GPCR, its homologues and other structurally or functionally related genes from human and other species such as mouse, pig, sheep, etc to be accomplished. Polynucleotides which are identical or sufficiently identical to a nucleotide sequence contained in SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 4, SEQ ID NO: 6, SEQ ID NO: 7, SEQ ID NO: 8, SEQ ID NO: 10, SEQ ID

NO: 12, SEQ ID NO: 13, SEQ ID NO: 15, SEQ ID NO: 16 or SEQ ID NO: 18, or a fragment thereof, may be used as hybridization probes for cDNA and genomic DNA, to isolate partial or full-length cDNAs and genomic clones encoding Conrad GPCR from appropriate libraries. Such probes may also be used to isolate cDNA and genomic clones of other genes (including genes encoding homologues and orthologues from species other than human) that have sequence similarity, preferably high sequence similarity, to the Conrad GPCR gene. Hybridization screening, cloning and sequencing techniques are known to those of skill in the art and are described in, for example, Sambrook et al (*supra*).

Typically nucleotide sequences suitable for use as probes are 70% identical, preferably 80% identical, more preferably 90% identical, even more preferably 95% identical to that of the referent. The probes generally will comprise at least 15 nucleotides. Preferably, such probes will have at least 30 nucleotides and may have at least 50 nucleotides. Particularly preferred probes will range between 150 and 500 nucleotides, more particularly about 300 nucleotides.

In one embodiment, to obtain a polynucleotide encoding a Conrad GPCR polypeptide, including homologues and orthologues from species other than human, comprises the steps of screening an appropriate library under stringent hybridization conditions with a labelled probe having the SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 4, SEQ ID NO: 6, SEQ ID NO: 7, SEQ ID NO: 8, SEQ ID NO: 10, SEQ ID NO: 12, SEQ ID NO: 13, SEQ ID NO: 15, SEQ ID NO: 16 or SEQ ID NO: 18, or a fragment thereof and isolating partial or full-length cDNA and genomic clones containing said polynucleotide sequence. Such hybridization techniques are well known to those of skill in the art. Stringent hybridization conditions are as defined above or alternatively conditions under overnight incubation at 42 degrees C. in a solution comprising: 50% formamide, 5XSSC (150 mM NaCl, 15mM trisodium citrate), 50 mM sodium phosphate (pH7.6), 5XDenhardt's solution, 10% dextran sulphate, and 20 microgram/ml denatured, sheared salmon sperm DNA, followed by washing the filters in 0.1XSSC at about 65 degrees C.

Functional Assay for Conrad GPCR

The cloned putative Conrad GPCR polynucleotides may be verified by sequence analysis or functional assays. For example, the putative Conrad GPCR or homologue may be assayed for receptor activity as follows. Capped RNA transcripts from linearized plasmid templates encoding the Conrad receptor cDNAs are synthesized *in vitro* with RNA polymerases in accordance with standard procedures. *In vitro* transcripts are suspended in water at a final concentration of 0.2 mg/ml. Ovarian lobes are removed from adult female toads, Stage V defolliculated oocytes are obtained, and RNA transcripts (10 ng/oocyte) are injected in a 50 nl bolus using a microinjection apparatus. Two electrode voltage clamps are used to measure the currents from individual *Xenopus* oocytes in response to agonist exposure. Recordings are made in Ca^{2+} free Barth's medium at room temperature. The *Xenopus* system may also be used to screen known ligands and tissue/cell extracts for activating ligands, as described in further detail below.

Expression Assays for Conrad GPCR

In order to design useful therapeutics for treating Conrad GPCR associated diseases, it is useful to determine the expression profile of Conrad (whether wild-type or a particular mutant). Thus, methods known in the art may be used to determine the organs, tissues and cell types (as well as the developmental stages) in which Conrad is expressed. For example, traditional or "electronic" Northerns may be conducted. Reverse-transcriptase PCR (RT-PCR) may also be employed to assay expression of the Conrad gene or mutant. More sensitive methods for determining the expression profile of Conrad include RNAse protection assays, as known in the art.

Northern analysis is a laboratory technique used to detect the presence of a transcript of a gene and involves the hybridization of a labeled nucleotide sequence to a membrane on which RNAs from a particular cell type or tissue have been bound. (Sambrook, supra, ch. 7 and Ausubel, F. M. et al. supra, ch. 4 and 16.) Analogous computer techniques ("electronic Northerns") applying BLAST may be used to search for

identical or related molecules in nucleotide databases such as GenBank or the LIFESEQ database (Incyte Pharmaceuticals). This type of analysis has advantages in that they may be faster than multiple membrane-based hybridizations. In addition, the sensitivity of the computer search can be modified to determine whether any particular match is categorized as exact or homologous.

The polynucleotides and polypeptides, including the probes described above, may be employed as research reagents and materials for discovery of treatments and diagnostics to animal and human disease, as explained in further detail elsewhere in this document.

EXPRESSION OF CONRAD GPCR POLYPEPTIDES

We further describe a process for producing a Conrad GPCR polypeptide. The method comprises in general culturing a host cell comprising a nucleic acid encoding Conrad GPCR polypeptide, or a homologue, variant, or derivative thereof, under suitable conditions (i.e., conditions in which the Conrad GPCR polypeptide is expressed).

In order to express a biologically active Conrad GPCR, the nucleotide sequences encoding Conrad GPCR or homologues, variants, or derivatives thereof are inserted into appropriate expression vector, i.e., a vector which contains the necessary elements for the transcription and translation of the inserted coding sequence.

Methods which are well known to those skilled in the art are used to construct expression vectors containing sequences encoding Conrad GPCR and appropriate transcriptional and translational control elements. These methods include *in vitro* recombinant DNA techniques, synthetic techniques, and in vivo genetic recombination. Such techniques are described in Sambrook, J. et al. (1989; Molecular Cloning, A Laboratory Manual, ch. 4, 8, and 16-17, Cold Spring Harbor Press, Plainview, N.Y.) and Ausubel, F. M. et al. (1995 and periodic supplements; Current Protocols in Molecular Biology, ch. 9, 13, and 16, John Wiley & Sons, New York, N.Y.).

A variety of expression vector/host systems may be utilized to contain and express sequences encoding Conrad GPCR. These include, but are not limited to, microorganisms such as bacteria transformed with recombinant bacteriophage, plasmid, or cosmid DNA expression vectors; yeast transformed with yeast expression vectors; insect cell systems infected with virus expression vectors (e.g., baculovirus); plant cell systems transformed with virus expression vectors (e.g., cauliflower mosaic virus (CaMV) or tobacco mosaic virus (TMV)) or with bacterial expression vectors (e.g., Ti or pBR322 plasmids); or animal cell systems. Any suitable type of host cell may be employed.

The "control elements" or "regulatory sequences" are those non-translated regions of the vector (i.e., enhancers, promoters, and 5' and 3' untranslated regions) which interact with host cellular proteins to carry out transcription and translation. Such elements may vary in their strength and specificity. Depending on the vector system and host utilized, any number of suitable transcription and translation elements, including constitutive and inducible promoters, may be used. For example, when cloning in bacterial systems, inducible promoters such as the hybrid lacZ promoter of the BLUESCRIPT phagemid (Stratagene, La Jolla, Calif.) or PSPORT1 plasmid (GIBCO/BRL), and the like, may be used. The baculovirus polyhedrin promoter may be used in insect cells. Promoters or enhancers derived from the genomes of plant cells (e.g., heat shock, RUBISCO, and storage protein genes) or from plant viruses (e.g., viral promoters or leader sequences) may be cloned into the vector. In mammalian cell systems, promoters from mammalian genes or from mammalian viruses are preferable. If it is necessary to generate a cell line that contains multiple copies of the sequence encoding Conrad GPCR, vectors based on SV40 or EBV may be used with an appropriate selectable marker.

In bacterial systems, a number of expression vectors may be selected depending upon the use intended for Conrad GPCR. For example, when large quantities of Conrad GPCR are needed for the induction of antibodies, vectors which direct high level expression of fusion proteins that are readily purified may be used. Such vectors include, but are not limited to, multifunctional E. coli cloning and expression vectors such as BLUESCRIPT (Stratagene), in which the sequence encoding Conrad GPCR may be

ligated into the vector in frame with sequences for the amino-terminal Met and the subsequent 7 residues of β-galactosidase so that a hybrid protein is produced, pIN vectors (Van Heeke, G. and S. M. Schuster (1989) J. Biol. Chem. 264:5503-5509), and the like. pGEX vectors (Promega, Madison, Wis.) may also be used to express foreign polypeptides as fusion proteins with glutathione S-transferase (GST). In general, such fusion proteins are soluble and can easily be purified from lysed cells by adsorption to glutathione-agarose beads followed by elution in the presence of free glutathione. Proteins made in such systems may be designed to include heparin, thrombin, or factor XA protease cleavage sites so that the cloned polypeptide of interest can be released from the GST moiety at will.

In the yeast *Saccharomyces cerevisiae*, a number of vectors containing constitutive or inducible promoters, such as alpha factor, alcohol oxidase, and PGH, may be used. For reviews, see Ausubel (supra) and Grant et al. (1987; Methods Enzymol. 153:516-544).

In cases where plant expression vectors are used, the expression of sequences encoding Conrad GPCR may be driven by any of a number of promoters. For example, viral promoters such as the 35S and 19S promoters of CaMV may be used alone or in combination with the omega leader sequence from TMV. (Takamatsu, N. (1987) EMBO J. 6:307-311.) Alternatively, plant promoters such as the small subunit of RUBISCO or heat shock promoters may be used. (Coruzzi, G. et al. (1984) EMBO J. 3:1671-1680; Broglie, R. et al. (1984) Science 224:838-843; and Winter, J. et al. (1991) Results Probl. Cell Differ. 17:85-105.) These constructs can be introduced into plant cells by direct DNA transformation or pathogen-mediated transfection. Such techniques are described in a number of generally available reviews. (See, for example, Hobbs, S. or Murry, L. E. in McGraw Hill Yearbook of Science and Technology (1992) McGraw Hill, New York, N.Y.; pp. 191-196.).

An insect system may also be used to express Conrad GPCR. For example, in one such system, *Autographa californica* nuclear polyhedrosis virus (AcNPV) is used as a vector to express foreign genes in *Spodoptera frugiperda* cells or in *Trichoplusia* larvae.

The sequences encoding Conrad GPCR may be cloned into a non-essential region of the virus, such as the polyhedrin gene, and placed under control of the polyhedrin promoter. Successful insertion of Conrad GPCR will render the polyhedrin gene inactive and produce recombinant virus lacking coat protein. The recombinant viruses may then be used to infect, for example, *S. frugiperda* cells or *Trichoplusia* larvae in which Conrad GPCR may be expressed. (Engelhard, E. K. et al. (1994) Proc. Nat. Acad. Sci. 91:3224-3227.)

In mammalian host cells, a number of viral-based expression systems may be utilized. In cases where an adenovirus is used as an expression vector, sequences encoding Conrad GPCR may be ligated into an adenovirus transcription/translation complex consisting of the late promoter and tripartite leader sequence. Insertion in a non-essential E1 or E3 region of the viral genome may be used to obtain a viable virus which is capable of expressing Conrad GPCR in infected host cells. (Logan, J. and T. Shenk (1984) Proc. Natl. Acad. Sci. 81:3655-3659.) In addition, transcription enhancers, such as the Rous sarcoma virus (RSV) enhancer, may be used to increase expression in mammalian host cells.

Thus, for example, the Conrad receptors are expressed in either human embryonic kidney 293 (HEK293) cells or adherent dhfr CHO cells. To maximize receptor expression, typically all 5' and 3' untranslated regions (UTRs) are removed from the receptor cDNA prior to insertion into a pCDN or pCDNA3 vector. The cells are transfected with individual receptor cDNAs by lipofectin and selected in the presence of 400 mg/ml G418. After 3 weeks of selection, individual clones are picked and expanded for further analysis. HEK293 or CHO cells transfected with the vector alone serve as negative controls. To isolate cell lines stably expressing the individual receptors, about 24 clones are typically selected and analyzed by Northern blot analysis. Receptor mRNAs are generally detectable in about 50% of the G418-resistant clones analyzed.

Human artificial chromosomes (HACs) may also be employed to deliver larger fragments of DNA than can be contained and expressed in a plasmid. HACs of about 6 kb

to 10 Mb are constructed and delivered via conventional delivery methods (liposomes, polycationic amino polymers, or vesicles) for therapeutic purposes.

Specific initiation signals may also be used to achieve more efficient translation of sequences encoding Conrad GPCR. Such signals include the ATG initiation codon and adjacent sequences. In cases where sequences encoding Conrad GPCR and its initiation codon and upstream sequences are inserted into the appropriate expression vector, no additional transcriptional or translational control signals may be needed. However, in cases where only coding sequence, or a fragment thereof, is inserted, exogenous translational control signals including the ATG initiation codon should be provided. Furthermore, the initiation codon should be in the correct reading frame to ensure translation of the entire insert. Exogenous translational elements and initiation codons may be of various origins, both natural and synthetic. The efficiency of expression may be enhanced by the inclusion of enhancers appropriate for the particular cell system used, such as those described in the literature. (Scharf, D. et al. (1994) Results Probl. Cell Differ. 20:125-162.)

In addition, a host cell strain may be chosen for its ability to modulate expression of the inserted sequences or to process the expressed protein in the desired fashion. Such modifications of the polypeptide include, but are not limited to, acetylation, carboxylation, glycosylation, phosphorylation, lipidation, and acylation. Post-translational processing which cleaves a "prepro" form of the protein may also be used to facilitate correct insertion, folding, and/or function. Different host cells which have specific cellular machinery and characteristic mechanisms for post-translational activities (e.g., CHO, HeLa, MDCK, HEK293, and WI38), are available from the American Type Culture Collection (ATCC, Bethesda, Md.) and may be chosen to ensure the correct modification and processing of the foreign protein.

For long term, high yield production of recombinant proteins, stable expression is preferred. For example, cell lines capable of stably expressing Conrad GPCR can be transformed using expression vectors which may contain viral origins of replication and/or

endogenous expression elements and a selectable marker gene on the same or on a separate vector. Following the introduction of the vector, cells may be allowed to grow for about 1 to 2 days in enriched media before being switched to selective media. The purpose of the selectable marker is to confer resistance to selection, and its presence allows growth and recovery of cells which successfully express the introduced sequences. Resistant clones of stably transformed cells may be proliferated using tissue culture techniques appropriate to the cell type.

Any number of selection systems may be used to recover transformed cell lines. These include, but are not limited to, the herpes simplex virus thymidine kinase genes (Wigler, M. et al. (1977) Cell 11:223-32) and adenine phosphoribosyltransferase genes (Lowy, I. et al. (1980) Cell 22:817-23), which can be employed in tk^- or apr^- cells, respectively. Also, antimetabolite, antibiotic, or herbicide resistance can be used as the basis for selection. For example, dhfr confers resistance to methotrexate (Wigler, M. et al. (1980) Proc. Natl. Acad. Sci. 77:3567-70); npt confers resistance to the aminoglycosides neomycin and G-418 (Colbere-Garapin, F. et al (1981) J. Mol. Biol. 150:1-14); and als or pat confer resistance to chlorsulfuron and phosphinotricin acetyltransferase, respectively (Murry, supra). Additional selectable genes have been described, for example, trpB, which allows cells to utilize indole in place of tryptophan, or hisD, which allows cells to utilize histinol in place of histidine. (Hartman, S. C. and R. C. Mulligan (1988) Proc. Natl. Acad. Sci. 85:8047-51.) Recently, the use of visible markers has gained popularity with such markers as anthocyanins, β-glucuronidase and its substrate GUS, and luciferase and its substrate luciferin. These markers can be used not only to identify transformants, but also to quantify the amount of transient or stable protein expression attributable to a specific vector system. (Rhodes, C. A. et al. (1995) Methods Mol. Biol. 55:121-131.)

Although the presence/absence of marker gene expression suggests that the gene of interest is also present, the presence and expression of the gene may need to be confirmed. For example, if the sequence encoding Conrad GPCR is inserted within a marker gene sequence, transformed cells containing sequences encoding Conrad GPCR can be identified by the absence of marker gene function. Alternatively, a marker gene can be

placed in tandem with a sequence encoding Conrad GPCR under the control of a single promoter. Expression of the marker gene in response to induction or selection usually indicates expression of the tandem gene as well.

Alternatively, host cells which contain the nucleic acid sequence encoding Conrad GPCR and express Conrad GPCR may be identified by a variety of procedures known to those of skill in the art. These procedures include, but are not limited to, DNA--DNA or DNA-RNA hybridizations and protein bioassay or immunoassay techniques which include membrane, solution, or chip based technologies for the detection and/or quantification of nucleic acid or protein sequences.

The presence of polynucleotide sequences encoding Conrad GPCR can be detected by DNA--DNA or DNA-RNA hybridization or amplification using probes or fragments or fragments of polynucleotides encoding Conrad GPCR. Nucleic acid amplification based assays involve the use of oligonucleotides or oligomers based on the sequences encoding Conrad GPCR to detect transformants containing DNA or RNA encoding Conrad GPCR.

A variety of protocols for detecting and measuring the expression of Conrad GPCR, using either polyclonal or monoclonal antibodies specific for the protein, are known in the art. Examples of such techniques include enzyme-linked immunosorbent assays (ELISAs), radioimmunoassays (RIAs), and fluorescence activated cell sorting (FACS). A two-site, monoclonal-based immunoassay utilizing monoclonal antibodies reactive to two non-interfering epitopes on Conrad GPCR is preferred, but a competitive binding assay may be employed. These and other assays are well described in the art, for example, in Hampton, R. et al. (1990; Serological Methods, a Laboratory Manual, Section IV, APS Press, St Paul, Minn.) and in Maddox, D. E. et al. (1983; J. Exp. Med. 158:1211-1216).

A wide variety of labels and conjugation techniques are known by those skilled in the art and may be used in various nucleic acid and amino acid assays. Means for producing labeled hybridization or PCR probes for detecting sequences related to

polynucleotides encoding Conrad GPCR include oligolabeling, nick translation, end-labeling, or PCR amplification using a labeled nucleotide. Alternatively, the sequences encoding Conrad GPCR, or any fragments thereof, may be cloned into a vector for the production of an mRNA probe. Such vectors are known in the art, are commercially available, and may be used to synthesize RNA probes in vitro by addition of an appropriate RNA polymerase such as T7, T3, or SP6 and labeled nucleotides. These procedures may be conducted using a variety of commercially available kits, such as those provided by Pharmacia & Upjohn (Kalamazoo, Mich.), Promega (Madison, Wis.), and U.S. Biochemical Corp. (Cleveland, Ohio). Suitable reporter molecules or labels which may be used for ease of detection include radionuclides, enzymes, fluorescent, chemiluminescent, or chromogenic agents, as well as substrates, cofactors, inhibitors, magnetic particles, and the like.

Host cells transformed with nucleotide sequences encoding Conrad GPCR may be cultured under conditions suitable for the expression and recovery of the protein from cell culture. The protein produced by a transformed cell may be located in the cell membrane, secreted or contained intracellularly depending on the sequence and/or the vector used. As will be understood by those of skill in the art, expression vectors containing polynucleotides which encode Conrad GPCR may be designed to contain signal sequences which direct secretion of Conrad GPCR through a prokaryotic or eukaryotic cell membrane. Other constructions may be used to join sequences encoding Conrad GPCR to nucleotide sequences encoding a polypeptide domain which will facilitate purification of soluble proteins. Such purification facilitating domains include, but are not limited to, metal chelating peptides such as histidine-tryptophan modules that allow purification on immobilized metals, protein A domains that allow purification on immobilized immunoglobulin, and the domain utilized in the FLAGS extension/affinity purification system (Immunex Corp., Seattle, Wash.). The inclusion of cleavable linker sequences, such as those specific for Factor XA or enterokinase (Invitrogen, San Diego, Calif.), between the purification domain and the Conrad GPCR encoding sequence may be used to facilitate purification. One such expression vector provides for expression of a fusion protein containing Conrad GPCR and a nucleic acid encoding 6 histidine residues

preceding a thioredoxin or an enterokinase cleavage site. The histidine residues facilitate purification on immobilized metal ion affinity chromatography (IMIAC; described in Porath, J. et al. (1992) Prot. Exp. Purif. 3: 263-281), while the enterokinase cleavage site provides a means for purifying Conrad GPCR from the fusion protein. A discussion of vectors which contain fusion proteins is provided in Kroll, D. J. et al. (1993; DNA Cell Biol. 12:441-453).

Fragments of Conrad GPCR may be produced not only by recombinant production, but also by direct peptide synthesis using solid-phase techniques. (Merrifield J. (1963) J. Am. Chem. Soc. 85:2149-2154.) Protein synthesis may be performed by manual techniques or by automation. Automated synthesis may be achieved, for example, using the Applied Biosystems 431A peptide synthesizer (Perkin Elmer). Various fragments of Conrad GPCR may be synthesized separately and then combined to produce the full length molecule.

BIOSENSORS

The Conrad polypeptides, nucleic acids, probes, antibodies, expression vectors and ligands are useful as (and for the production of) biosensors.

According to Aizawa (1988), *Anal. Chem. Symp.* 17: 683, a biosensor is defined as being a unique combination of a receptor for molecular recognition, for example a selective layer with immobilized antibodies or receptors such as a Conrad G-protein coupled receptor, and a transducer for transmitting the values measured. One group of such biosensors will detect the change which is caused in the optical properties of a surface layer due to the interaction of the receptor with the surrounding medium. Among such techniques may be mentioned especially ellipso-metry and surface plasmon resonance. Biosensors incorporating Conrad may be used to detect the presence or level of Conrad ligands, for example, nucleotides such as purines or purine analogues, or analogues of these ligands. The construction of such biosensors is well known in the art.

Thus, cell lines expressing Conrad receptor may be used as reporter systems for detection of ligands such as ATP via receptor-promoted formation of [3H]inositol phosphates or other second messengers (Watt et al., 1998, *J Biol Chem* May 29;273(22):14053-8). Receptor-ligand biosensors are also described in Hoffman et al., 2000, *Proc Natl Acad Sci U S A* Oct 10;97(21):11215-20. Optical and other biosensors comprising Conrad may also be used to detect the level or presence of interaction with G-proteins and other proteins, as described by, for example, Figler et al, 1997, *Biochemistry* Dec 23;36(51):16288-99 and Sarrio et al., 2000, *Mol Cell Biol* 2000 Jul;20(14):5164-74). Sensor units for biosensors are described in, for example, US 5,492,840.

SCREENING ASSAYS

The Conrad GPCR polypeptide, including homologues, variants, and derivatives, whether natural or recombinant, may be employed in a screening process for compounds which bind the receptor and which activate (agonists) or inhibit activation of (antagonists) of Conrad. Thus, such polypeptides may also be used to assess the binding of small molecule substrates and ligands in, for example, cells, cell-free preparations, chemical libraries, and natural product mixtures. These substrates and ligands may be natural substrates and ligands or may be structural or functional mimetics. See Coligan et al., *Current Protocols in Immunology* 1(2):Chapter 5 (1991).

Conrad GPCR polypeptides are responsible for many biological functions, including many pathologies. Accordingly, it is desirous to find compounds and drugs which stimulate Conrad GPCR on the one hand and which can inhibit the function of Conrad GPCR on the other hand. In general, agonists and antagonists are employed for therapeutic and prophylactic purposes for such conditions as infections such as bacterial, fungal, protozoan and viral infections, particularly infections caused by HIV-1 or HIV-2; pain; cancers; diabetes, obesity; anorexia; bulimia; asthma; Parkinson's disease; thrombosis; acute heart failure; hypotension; hypertension; erectile dysfunction; urinary retention; metabolic bone diseases such as osteoporisis and osteo petrosis; angina pectoris; myocardial infarction; ulcers; asthma; allergies; rheumatoid arthritis; inflammatory bowel

disease; irritable bowel syndrome benign prostatic hypertrophy; and psychotic and neurological disorders, including anxiety, schizophrenia, manic depression, delirium, dementia, severe mental retardation and dyskinesias, such as Huntington's disease or Gilles dela Tourett's syndrome.

In particular, agonists and antagonists of Conrad GPCR may be used to treat or prevent long QT syndrome-4 with sinus bradycardia disease, mental health wellness-2 disease, psoriasis or susceptibility to psoriasis, dentin dysplasia, type II disease and neutropenia, neonatal alloimmune disease.

Rational design of candidate compounds likely to be able to interact with Conrad GPCR protein may be based upon structural studies of the molecular shapes of a polypeptide as described here. One means for determining which sites interact with specific other proteins is a physical structure determination, e.g., X-ray crystallography or two-dimensional NMR techniques. These will provide guidance as to which amino acid residues form molecular contact regions. For a detailed description of protein structural determination, see, e.g., Blundell and Johnson (1976) *Protein Crystallography*, Academic Press, New York.

An alternative to rational design uses a screening procedure which involves in general producing appropriate cells which express the Conrad receptor polypeptide on the surface thereof. Such cells include cells from animals, yeast, *Drosophila* or *E. coli*. Cells expressing the receptor (or cell membrane containing the expressed receptor) are then contacted with a test compound to observe binding, or stimulation or inhibition of a functional response. For example, *Xenopus* oocytes may be injected with Conrad mRNA or polypeptide, and currents induced by exposure to test compounds measured by use of voltage clamps measured, as described in further detail elsewhere.

Furthermore, microphysiometric assays may be employed to assay Conrad receptor activity. Activation of a wide variety of secondary messenger systems results in extrusion of small amounts of acid from a cell. The acid formed is largely as a result of the increased

metabolic activity required to fuel the intracellular signalling process. The pH changes in the media surrounding the cell are very small but are detectable by, for example, the CYTOSENSOR microphysiometer (Molecular Devices Ltd., Menlo Park, Calif.). The CYTOSENSOR is thus capable of detecting the activation of a receptor which is coupled to an energy utilizing intracellular signaling pathway such as the G-protein coupled receptor.

Instead of testing each candidate compound individually with the Conrad receptor, a library or bank of candidate ligands may advantageously be produced and screened. Thus, for example, a bank of over 200 putative receptor ligands has been assembled for screening. The bank comprises: transmitters, hormones and chemokines known to act via a human seven transmembrane (7TM) receptor; naturally occurring compounds which may be putative agonists for a human 7TM receptor, non-mammalian, biologically active peptides for which a mammalian counterpart has not yet been identified; and compounds not found in nature, but which activate 7TM receptors with unknown natural ligands. This bank is used to screen the receptor for known ligands, using both functional (i.e. calcium, cAMP, microphysiometer, oocyte electrophysiology, etc, see elsewhere) as well as binding assays as described in further detail elsewhere. However, a large number of mammalian receptors exist for which there remains, as yet, no cognate activating ligand (agonist) or deactivating ligand (antagonist). Thus, active ligands for these receptors may not be included within the ligands banks as identified to date. Accordingly, the Conrad receptor is also functionally screened (using calcium, cAMP, microphysiometer, ooyte electrophysiology, etc., functional screens) against tissue extracts to identify natural ligands. Extracts that produce positive functional responses can be sequentially subfractionated, with the fractions being assayed as described here, until an activating ligand is isolated and identified.

7TM receptors which are expressed in HEK 293 cells have been shown to be coupled functionally to activation of PLC and calcium mobilization and/or cAMP stimuation or inhibition. One screening technique therefore includes the use of cells which express the Conrad GPCR receptor (for example, transfected *Xenopus* oocytes, CHO or

HEK293 cells) in a system which measures extracellular pH or intracellular calcium changes caused by receptor activation. In this technique, compounds may be contacted with cells expressing the receptor polypeptide. A second messenger response, e.g., signal transduction, pH changes, or changes in calcium level, is then measured to determine whether the potential compound activates or inhibits the receptor.

In such experiments, basal calcium levels in the HEK 293 cells in receptor-transfected or vector control cells are observed to be in the normal, 100 nM to 200 nM, range. HEK 293 cells expressing Conrad GPCR or recombinant Conrad GPCR are loaded with fura 2 and in a single day more than 150 selected ligands or tissue/cell extracts are evaluated for agonist induced calcium mobilization. Similarly, HEK 293 cells expressing Conrad GPCR or recombinant Conrad GPCR are evaluated for the stimulation or inhibition of cAMP production using standard cAMP quantitation assays. Agonists presenting a calcium transient or cAMP fluctuation are tested in vector control cells to determine if the response is unique to the transfected cells expressing receptor.

Another method involves screening for receptor inhibitors by determining inhibition or stimulation of Conrad receptor-mediated cAMP and/or adenylate cyclase accumulation. Such a method involves transfecting a eukaryotic cell with the receptor as described here to express the receptor on the cell surface. The cell is then exposed to potential antagonists in the presence of the receptor. The amount of cAMP accumulation is then measured. If the potential antagonist binds the receptor, and thus inhibits receptor binding, the levels of receptor-mediated cAMP, or adenylate cyclase, activity will be reduced or increased.

Another method for detecting agonists or antagonists for the receptor is the yeast based technology as described in U.S. Pat. No. 5,482,835, incorporated by reference herein.

Where the candidate compounds are proteins, in particular antibodies or peptides, libraries of candidate compounds may be screened using phage display techniques. Phage

display is a protocol of molecular screening which utilises recombinant bacteriophage. The technology involves transforming bacteriophage with a gene that encodes one compound from the library of candidate compounds, such that each phage or phagemid expresses a particular candidate compound. The transformed bacteriophage (which preferably is tethered to a solid support) expresses the appropriate candidate compound and displays it on their phage coat. Specific candidate compounds which are capable of binding to a polypeptide or peptide as described here are enriched by selection strategies based on affinity interaction. The successful candidate agents are then characterised. Phage display has advantages over standard affinity ligand screening technologies. The phage surface displays the candidate agent in a three dimensional configuration, more closely resembling its naturally occurring conformation. This allows for more specific and higher affinity binding for screening purposes.

Another method of screening a library of compounds utilises eukaryotic or prokaryotic host cells which are stably transformed with recombinant DNA molecules expressing a library of compounds. Such cells, either in viable or fixed form, can be used for standard binding-partner assays. See also Parce *et al.* (1989) Science 246:243-247; and Owicki *et al.* (1990) Proc. Nat'l Acad. Sci. USA 87;4007-4011, which describe sensitive methods to detect cellular responses. Competitive assays are particularly useful, where the cells expressing the library of compounds are contacted or incubated with a labelled antibody known to bind to a Conrad polypeptide, such as ^{125}I-antibody, and a test sample such as a candidate compound whose binding affinity to the binding composition is being measured. The bound and free labelled binding partners for the polypeptide are then separated to assess the degree of binding. The amount of test sample bound is inversely proportional to the amount of labelled antibody binding to the polypeptide.

Any one of numerous techniques can be used to separate bound from free binding partners to assess the degree of binding. This separation step could typically involve a procedure such as adhesion to filters followed by washing, adhesion to plastic following by washing, or centrifugation of the cell membranes.

Still another approach is to use solubilized, unpurified or solubilized purified polypeptide or peptides, for example extracted from transformed eukaryotic or prokaryotic host cells. This allows for a "molecular" binding assay with the advantages of increased specificity, the ability to automate, and high drug test throughput.

Another technique for candidate compound screening involves an approach which provides high throughput screening for new compounds having suitable binding affinity, e.g., to a polypeptide as described here, and is described in detail in International Patent application no. WO 84/03564 (Commonwealth Serum Labs.), published on September 13 1984. First, large numbers of different small peptide test compounds are synthesized on a solid substrate, e.g., plastic pins or some other appropriate surface; see Fodor *et al.* (1991). Then all the pins are reacted with solubilized polypeptide and washed. The next step involves detecting bound polypeptide. Compounds which interact specifically with the polypeptide will thus be identified.

Ligand binding assays provide a direct method for ascertaining receptor pharmacology and are adaptable to a high throughput format. The purified ligand for a receptor may be radiolabeled to high specific activity (50-2000 Ci/mmol) for binding studies. A determination is then made that the process of radiolabeling does not diminish the activity of the ligand towards its receptor. Assay conditions for buffers, ions, pH and other modulators such as nucleotides are optimized to establish a workable signal to noise ratio for both membrane and whole cell receptor sources. For these assays, specific receptor binding is defined as total associated radioactivity minus the radioactivity measured in the presence of an excess of unlabeled competing ligand. Where possible, more than one competing ligand is used to define residual nonspecific binding.

The assays may simply test binding of a candidate compound wherein adherence to the cells bearing the receptor is detected by means of a label directly or indirectly associated with the candidate compound or in an assay involving competition with a labeled competitor. Further, these assays may test whether the candidate compound results in a signal generated by activation of the receptor, using detection systems appropriate to

the cells bearing the receptor at their surfaces. Inhibitors of activation are generally assayed in the presence of a known agonist and the effect on activation by the agonist by the presence of the candidate compound is observed.

Further, the assays may simply comprise the steps of mixing a candidate compound with a solution containing a Conrad GPCR polypeptide to form a mixture, measuring Conrad GPCR activity in the mixture, and comparing the Conrad GPCR activity of the mixture to a standard.

The Conrad GPCR cDNA, protein and antibodies to the protein may also be used to configure assays for detecting the effect of added compounds on the production of Conrad GPCR mRNA and protein in cells. For example, an ELISA may be constructed for measuring secreted or cell associated levels of Conrad GPCR protein using monoclonal and polyclonal antibodies by standard methods known in the art, and this can be used to discover agents which may inhibit or enhance the production of Conrad GPCR (also called antagonist or agonist, respectively) from suitably manipulated cells or tissues. Standard methods for conducting screening assays are well understood in the art.

Examples of potential Conrad GPCR antagonists include antibodies or, in some cases, nucleotides and their analogues, including purines and purine analogues, oligonucleotides or proteins which are closely related to the ligand of the Conrad GPCR, e.g., a fragment of the ligand, or small molecules which bind to the receptor but do not elicit a response, so that the activity of the receptor is prevented.

We therefore also provide a compound capable of binding specifically to a Conrad polypeptide and/or peptide.

The term "compound" refers to a chemical compound (naturally occurring or synthesised), such as a biological macromolecule (e.g., nucleic acid, protein, non-peptide, or organic molecule), or an extract made from biological materials such as bacteria, plants,

fungi, or animal (particularly mammalian) cells or tissues, or even an inorganic element or molecule. Preferably the compound is an antibody.

The materials necessary for such screening to be conducted may be packaged into a screening kit. Such a screening kit is useful for identifying agonists, antagonists, ligands, receptors, substrates, enzymes, etc. for Conrad GPCR polypeptides or compounds which decrease or enhance the production of Conrad GPCR polypeptides. The screening kit comprises: (a) a Conrad GPCR polypeptide; (b) a recombinant cell expressing a Conrad GPCR polypeptide; (c) a cell membrane expressing a Conrad GPCR polypeptide; or (d) antibody to a Conrad GPCR polypeptide. The screening kit may optionally comprise instructions for use.

TRANSGENIC ANIMALS

We further describe transgenic animals capable of expressing natural or recombinant Conrad GPCR, or a homologue, variant or derivative, at elevated or reduced levels compared to the normal expression level. Included are transgenic animals ("Conrad knockout"s) which do not express functional Conrad receptor as a result of one or more loss of function mutations, including a deletion, of the Conrad gene. Preferably, such a transgenic animal is a non-human mammal, such as a pig, a sheep or a rodent. Most preferably the transgenic animal is a mouse or a rat. Such transgenic animals may be used in screening procedures to identify agonists and/or antagonists of Conrad GPCR, as well as to test for their efficacy as treatments for diseases *in vivo*.

For example, transgenic animals that have been engineered to be deficient in the production of Conrad GPCR may be used in assays to identify agonists and/or antagonists of Conrad GPCR. One assay is designed to evaluate a potential drug (aa candidate ligand or compound) to determine if it produces a physiological response in the absence of Conrad GPCR receptors. This may be accomplished by administering the drug to a transgenic animal as discussed above, and then assaying the animal for a particular response. Although any physiological parameter could be measured in this assay, preferred

responses include one or more of the following: changes to disease resistance; altered inflammatory responses; altered tumour susceptability: a change in blood pressure; neovascularization; a change in eating behavior; a change in body weight; a change in bone density; a change in body temperature; insulin secretion; gonadotropin secretion; nasal and bronchial secretion; vasoconstriction; loss of memory; anxiety; hyporeflexia or hyperreflexia; pain or stress responses.

Tissues derived from the Conrad knockout animals may be used in receptor binding assays to determine whether the potential drug (a candidate ligand or compound) binds to the Conrad receptor. Such assays can be conducted by obtaining a first receptor preparation from the transgenic animal engineered to be deficient in Conrad receptor production and a second receptor preparation from a source known to bind any identified Conrad ligands or compounds. In general, the first and second receptor preparations will be similar in all respects except for the source from which they are obtained. For example, if brain tissue from a transgenic animal (such as described above and below) is used in an assay, comparable brain tissue from a normal (wild type) animal is used as the source of the second receptor preparation. Each of the receptor preparations is incubated with a ligand known to bind to Conrad receptors, both alone and in the presence of the candidate ligand or compound. Preferably, the candidate ligand or compound will be examined at several different concentrations.

The extent to which binding by the known ligand is displaced by the test compound is determined for both the first and second receptor preparations. Tissues derived from transgenic animals may be used in assays directly or the tissues may be processed to isolate membranes or membrane proteins, which are themselves used in the assays. A preferred transgenic animal is the mouse. The ligand may be labeled using any means compatible with binding assays. This would include, without limitation, radioactive, enzymatic, fluorescent or chemiluminescent labeling (as well as other labelling techniques as described in further detail above).

Furthermore, antagonists of Conrad GPCR receptor may be identified by administering candidate compounds, etc, to wild type animals expressing functional Conrad, and animals identified which exhibit any of the phenotypic characteristics associated with reduced or abolished expression of Conrad receptor function.

Detailed methods for generating non-human transgenic animal are described in further detail below. Transgenic gene constructs can be introduced into the germ line of an animal to make a transgenic mammal. For example, one or several copies of the construct may be incorporated into the genome of a mammalian embryo by standard transgenic techniques.

In an exemplary embodiment, the transgenic non-human animals as described here are produced by introducing transgenes into the germline of the non-human animal. Embryonal target cells at various developmental stages can be used to introduce transgenes. Different methods are used depending on the stage of development of the embryonal target cell. The specific line(s) of any animal used to do so are selected for general good health, good embryo yields, good pronuclear visibility in the embryo, and good reproductive fitness. In addition, the haplotype is a significant factor.

Introduction of the transgene into the embryo can be accomplished by any means known in the art such as, for example, microinjection, electroporation, or lipofection. For example, the Conrad receptor transgene can be introduced into a mammal by microinjection of the construct into the pronuclei of the fertilized mammalian egg(s) to cause one or more copies of the construct to be retained in the cells of the developing mammal(s). Following introduction of the transgene construct into the fertilized egg, the egg may be incubated in vitro for varying amounts of time, or reimplanted into the surrogate host, or both. *In vitro* incubation to maturity is within the scope of this document. One common method in to incubate the embryos in vitro for about 1-7 days, depending on the species, and then reimplant them into the surrogate host.

The progeny of the transgenically manipulated embryos can be tested for the presence of the construct by Southern blot analysis of the segment of tissue. If one or more copies of the exogenous cloned construct remains stably integrated into the genome of such transgenic embryos, it is possible to establish permanent transgenic mammal lines carrying the transgenically added construct.

The litters of transgenically altered mammals can be assayed after birth for the incorporation of the construct into the genome of the offspring. Preferably, this assay is accomplished by hybridizing a probe corresponding to the DNA sequence coding for the desired recombinant protein product or a segment thereof onto chromosomal material from the progeny. Those mammalian progeny found to contain at least one copy of the construct in their genome are grown to maturity.

For the purposes of this document a zygote is essentially the formation of a diploid cell which is capable of developing into a complete organism. Generally, the zygote will be comprised of an egg containing a nucleus formed, either naturally or artificially, by the fusion of two haploid nuclei from a gamete or gametes. Thus, the gamete nuclei must be ones which are naturally compatible, i.e., ones which result in a viable zygote capable of undergoing differentiation and developing into a functioning organism. Generally, a euploid zygote is preferred. If an aneuploid zygote is obtained, then the number of chromosomes should not vary by more than one with respect to the euploid number of the organism from which either gamete originated.

In addition to similar biological considerations, physical ones also govern the amount (e.g., volume) of exogenous genetic material which can be added to the nucleus of the zygote or to the genetic material which forms a part of the zygote nucleus. If no genetic material is removed, then the amount of exogenous genetic material which can be added is limited by the amount which will be absorbed without being physically disruptive. Generally, the volume of exogenous genetic material inserted will not exceed about 10 picoliters. The physical effects of addition must not be so great as to physically destroy the viability of the zygote. The biological limit of the number and variety of DNA

sequences will vary depending upon the particular zygote and functions of the exogenous genetic material and will be readily apparent to one skilled in the art, because the genetic material, including the exogenous genetic material, of the resulting zygote must be biologically capable of initiating and maintaining the differentiation and development of the zygote into a functional organism.

The number of copies of the transgene constructs which are added to the zygote is dependent upon the total amount of exogenous genetic material added and will be the amount which enables the genetic transformation to occur. Theoretically only one copy is required; however, generally, numerous copies are utilized, for example, 1,000-20,000 copies of the transgene construct, in order to insure that one copy is functional. There will often be an advantage to having more than one functioning copy of each of the inserted exogenous DNA sequences to enhance the phenotypic expression of the exogenous DNA sequences.

Any technique which allows for the addition of the exogenous genetic material into nucleic genetic material can be utilized so long as it is not destructive to the cell, nuclear membrane or other existing cellular or genetic structures. The exogenous genetic material is preferentially inserted into the nucleic genetic material by microinjection. Microinjection of cells and cellular structures is known and is used in the art.

Reimplantation is accomplished using standard methods. Usually, the surrogate host is anesthetized, and the embryos are inserted into the oviduct. The number of embryos implanted into a particular host will vary by species, but will usually be comparable to the number of off spring the species naturally produces.

Transgenic offspring of the surrogate host may be screened for the presence and/or expression of the transgene by any suitable method. Screening is often accomplished by Southern blot or Northern blot analysis, using a probe that is complementary to at least a portion of the transgene. Western blot analysis using an antibody against the protein encoded by the transgene may be employed as an alternative or additional method for

screening for the presence of the transgene product. Typically, DNA is prepared from tail tissue and analyzed by Southern analysis or PCR for the transgene. Alternatively, the tissues or cells believed to express the transgene at the highest levels are tested for the presence and expression of the transgene using Southern analysis or PCR, although any tissues or cell types may be used for this analysis.

Alternative or additional methods for evaluating the presence of the transgene include, without limitation, suitable biochemical assays such as enzyme and/or immunological assays, histological stains for particular marker or enzyme activities, flow cytometric analysis, and the like. Analysis of the blood may also be useful to detect the presence of the transgene product in the blood, as well as to evaluate the effect of the transgene on the levels of various types of blood cells and other blood constituents.

Progeny of the transgenic animals may be obtained by mating the transgenic animal with a suitable partner, or by in vitro fertilization of eggs and/or sperm obtained from the transgenic animal. Where mating with a partner is to be performed, the partner may or may not be transgenic and/or a knockout; where it is transgenic, it may contain the same or a different transgene, or both. Alternatively, the partner may be a parental line. Where in vitro fertilization is used, the fertilized embryo may be implanted into a surrogate host or incubated in vitro, or both. Using either method, the progeny may be evaluated for the presence of the transgene using methods described above, or other appropriate methods.

The transgenic animals so produced will include exogenous genetic material. As set out above, the exogenous genetic material will, in certain embodiments, be a DNA sequence which results in the production of a Conrad GPCR receptor. Further, in such embodiments the sequence will be attached to a transcriptional control element, e.g., a promoter, which preferably allows the expression of the transgene product in a specific type of cell.

Retroviral infection can also be used to introduce transgene into a non-human animal. The developing non-human embryo can be cultured in vitro to the blastocyst stage. During this time, the blastomeres can be targets for retroviral infection (Jaenich, R. (1976) PNAS 73:1260-1264). Efficient infection of the blastomeres is obtained by enzymatic treatment to remove the zona pellucida (Manipulating the Mouse Embryo, Hogan eds. (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, 1986). The viral vector system used to introduce the transgene is typically a replication-defective retrovirus carrying the transgene (Jahner et al. (1985) PNAS 82:6927-6931; Van der Putten et al. (1985) PNAS 82:6148-6152). Transfection is easily and efficiently obtained by culturing the blastomeres on a monolayer of virus-producing cells (Van der Putten, supra; Stewart et al. (1987) EMBO J. 6:383-388). Alternatively, infection can be performed at a later stage. Virus or virus-producing cells can be injected into the blastocoele (Jahner et al. (1982) Nature 298:623-628). Most of the founders will be mosaic for the transgene since incorporation occurs only in a subset of the cells which formed the transgenic non-human animal. Further, the founder may contain various retroviral insertions of the transgene at different positions in the genome which generally will segregate in the offspring. In addition, it is also possible to introduce transgenes into the germ line by intrauterine retroviral infection of the midgestation embryo (Jahner et al. (1982) supra).

A third type of target cell for transgene introduction is the embryonal stem cell (ES). ES cells are obtained from pre-implantation embryos cultured in vitro and fused with embryos (Evans et al. (1981) Nature 292:154-156; Bradley et al. (1984) Nature 309:255-258; Gossler et al. (1986) PNAS 83: 9065-9069; and Robertson et al. (1986) Nature 322:445-448). Transgenes can be efficiently introduced into the ES cells by DNA transfection or by retrovirus-mediated transduction. Such transformed ES cells can thereafter be combined with blastocysts from a non-human animal. The ES cells thereafter colonize the embryo and contribute to the germ line of the resulting chimeric animal. For review see Jaenisch, R. (1988) Science 240:1468-1474.

We also provide non-human transgenic animals, where the transgenic animal is characterized by having an altered Conrad gene, preferably as described above, as models

for Conrad receptor function. Alterations to the gene include deletions or other loss of function mutations, introduction of an exogenous gene having a nucleotide sequence with targeted or random mutations, introduction of an exogenous gene from another species, or a combination thereof. The transgenic animals may be either homozygous or heterozygous for the alteration. The animals and cells derived therefrom are useful for screening biologically active agents that may modulate Conradreceptor function. The screening methods are of particular use for determining the specificity and action of potential therapies for infections such as bacterial, fungal, protozoan and viral infections, particularly infections caused by HIV-1 or HIV-2; pain; cancers; diabetes, obesity; anorexia; bulimia; asthma; Parkinson's disease; thrombosis; acute heart failure; hypotension; hypertension; erectile dysfunction; urinary retention; metabolic bone diseases such as osteoporisis and osteo petrosis; angina pectoris; myocardial infarction; ulcers; asthma; allergies; rheumatoid arthritis; inflammatory bowel disease; irritable bowel syndrome benign prostatic hypertrophy; and psychotic and neurological disorders, including anxiety, schizophrenia, manic depression, delirium, dementia, severe mental retardation and dyskinesias, such as Huntington's disease or Gilles dela Tourett's syndrome. The animals are useful as a model to investigate the role of Conrad receptors in normal brain, heart, spleen and liver function.

Another aspect pertains to a transgenic nonhuman animal having a functionally disrupted endogenous Conrad gene but which also carries in its genome, and expresses, a transgene encoding a heterologous Conrad protein (i.e., a Conrad from another species). Preferably, the animal is a mouse and the heterologous Conrad is a human Conrad. An animal, or cell lines derived from such an animal, which has been reconstituted with human Conrad, can be used to identify agents that inhibit human Conrad *in vivo* and *in vitro*. For example, a stimulus that induces signalling through human Conrad can be administered to the animal, or cell line, in the presence and absence of an agent to be tested and the response in the animal, or cell line, can be measured. An agent that inhibits human Conrad *in vivo* or *in vitro* can be identified based upon a decreased response in the presence of the agent compared to the response in the absence of the agent.

We also provide for a Conrad GPCR deficient transgenic non-human animal (a "Conrad GPCR knock-out"). Such an animal is one which expresses lowered or no Conrad GPCR activity, preferably as a result of an endogenous Conrad GPCR genomic sequence being disrupted or deleted. Preferably, such an animal expresses no GPCR activity. More preferably, the animal expresses no activity of the Conrad GPCR shown as SEQ ID NO: 3, SEQ ID NO: 5, SEQ ID NO: 9 or SEQ ID NO: 11. Conrad GPCR knock-outs may be generated by various means known in the art, as described in further detail below.

We further describe a nucleic acid construct for functionally disrupting a Conrad gene in a host cell. The nucleic acid construct comprises: a) a non-homologous replacement portion; b) a first homology region located upstream of the non-homologous replacement portion, the first homology region having a nucleotide sequence with substantial identity to a first Conrad gene sequence; and c) a second homology region located downstream of the non-homologous replacement portion, the second homology region having a nucleotide sequence with substantial identity to a second Conrad gene sequence, the second Conrad gene sequence having a location downstream of the first Conrad gene sequence in a naturally occurring endogenous Conrad gene. Additionally, the first and second homology regions are of sufficient length for homologous recombination between the nucleic acid construct and an endogenous Conrad gene in a host cell when the nucleic acid molecule is introduced into the host cell. In a preferred embodiment, the non-homologous replacement portion comprises an expression reporter, preferably including lacZ and a positive selection expression cassette, preferably including a neomycin phosphotransferase gene operatively linked to a regulatory element(s).

Preferably, the first and second Conrad gene sequences are derived from SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 4, SEQ ID NO: 6, SEQ ID NO: 7, SEQ ID NO: 8, SEQ ID NO: 10, SEQ ID NO: 12, SEQ ID NO: 13, SEQ ID NO: 15, SEQ ID NO: 16 or SEQ ID NO: 18,, or a homologue, variant or derivative thereof.

Another aspect pertains to recombinant vectors into which the nucleic acid construct as described here has been incorporated. Yet another aspect pertains to host cells

into which the nucleic acid construct has been introduced to thereby allow homologous recombination between the nucleic acid construct and an endogenous Conrad gene of the host cell, resulting in functional disruption of the endogenous Conrad gene. The host cell can be a mammalian cell that normally expresses Conrad from the liver, brain, spleen or heart, or a pluripotent cell, such as a mouse embryonic stem cell. Further development of an embryonic stem cell into which the nucleic acid construct has been introduced and homologously recombined with the endogenous Conrad gene produces a transgenic nonhuman animal having cells that are descendant from the embryonic stem cell and thus carry the Conrad gene disruption in their genome. Animals that carry the Conrad gene disruption in their germline can then be selected and bred to produce animals having the Conrad gene disruption in all somatic and germ cells. Such mice can then be bred to homozygosity for the Conrad gene disruption.

A Conrad GPCR deficient transgenic animal may be generated as follows:

Construction of Conrad Gene Targeting Vector

Murine Conrad genomic clones may be isolated from a mouse large insert PAC library obtained from HGMP (Hinxton, UK) using the human (SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 7 and SEQ ID NO: 8) or mouse (SEQ ID NO: 4, SEQ ID NO: 6, SEQ ID NO: 10 and SEQ ID NO: 12) open reading frame cDNA sequences, or a fragments of any of these, as a probe using standard techniques. The isolated murine Conrad genomic clones may then be restriction mapped in the region of the Conrad gene using small oligonucleotide probes and standard techniques. The murine genomic locus may be partially sequenced to enable the design of homologous arms to clone into the targeting vector.

The murine Conrad gene is a multi exon gene. A short (approx.1 kb) 5' homologous arm and a long (approx. 4 kb) 3' homologous arm were amplified by PCR and the fragment cloned into the targeting vector. The position of these arms is chosen to functionally disrupt the Conrad gene by deleting some or all of the seven transmembrane

spanning regions. A targeting vector is prepared where the deleted Conrad sequence is replaced with non-homologous sequences composed of an endogenous gene expression reporter (a fusion with a frame independent lacZ) upstream of a selection cassette composed of a self promoted neomycin phosphotransferase (neo) gene in the same orientation as the Conradgene.

Transfection and Analysis of Embryonal Stem Cells

Embryonal stem cells (Evans and Kaufman, 1981) are cultured on a neomycin resistant embryonal fibroblast feeder layer grown in Dulbecco's Modified Eagles medium supplemented with 20% Fetal Calf Serum, 10% new-born calf serum, 2 mM glutamine, non-essential amino acids, 100 μM 2-mercaptoethanol and 500 u/ml leukemia inhibitory factor. Medium is changed daily and ES cells are subcultured every three days. $5x10^6$ ES cells are transfected with 5 μg of linearized plasmid by electroporation (25 μF capacitance and 400 Volts). 24 hours following electroporation the transfected cells are cultured for 9 days in medium containing 200 μg/ml neomycin. Clones are picked into 96 well plates, replicated and expanded before being screened by PCR to identify clones in which homologous recombination had occurred between the endogenous Conrad gene and the targeting construct. From 200 picked clones several targets are identified. These clones were expanded to allow replicas to be frozen and sufficient high quality DNA to be prepared for Southern blot confirmation of the targeting event using external 5' and 3' probes, all using standard procedures (Russ et al, 2000)

Generation of Conrad GPCR Deficient Mice

C57BL/6 female and male mice are mated and blastocysts are isolated at 3.5 days of gestation. 10-12 cells from a chosen clone are injected per blastocyst and 7-8 blastocysts are implanted in the uterus of a pseudopregnant F1 female. A litter of chimeric pups are born of which some males are up to 100% agouti (indicating cells descendent from the targeted clone). Male chimeras are mated with female and MF1 and 129 mice,

and germline transmission is determined by the agouti coat color and by PCR genotyping respectively.

ANTIBODIES

For the purposes of this document, the term "antibody", unless specified to the contrary, includes but is not limited to, polyclonal, monoclonal, chimeric, single chain, Fab fragments and fragments produced by a Fab expression library. Such fragments include fragments of whole antibodies which retain their binding activity for a target substance, Fv, F(ab') and $F(ab')_2$ fragments, as well as single chain antibodies (scFv), fusion proteins and other synthetic proteins which comprise the antigen-binding site of the antibody. The antibodies and fragments thereof may be humanised antibodies, for example as described in EP-A-239400. Furthermore, antibodies with fully human variable regions (or their fragments), for example, as described in US Patent Nos. 5,545,807 and 6,075,181 may also be used. Neutralizing antibodies, i.e., those which inhibit biological activity of the substance amino acid sequences, are especially preferred for diagnostics and therapeutics.

Antibodies may be produced by standard techniques, such as by immunisation or by using a phage display library.

A polypeptide or peptide as described in this document may be used to develop an antibody by known techniques. Such an antibody may be capable of binding specifically to the Conrad GPCR protein or homologue, fragment, etc.

If polyclonal antibodies are desired, a selected mammal (e.g., mouse, rabbit, goat, horse, etc.) may be immunised with an immunogenic composition comprising such a polypeptide or peptide. Depending on the host species, various adjuvants may be used to increase immunological response. Such adjuvants include, but are not limited to, Freund's, mineral gels such as aluminium hydroxide, and surface active substances such as lysolecithin, pluronic polyols, polyanions, peptides, oil emulsions, keyhole limpet hemocyanin, and dinitrophenol. BCG (*Bacilli Calmette-Guerin*) and *Corynebacterium*

parvum are potentially useful human adjuvants which may be employed if purified the substance amino acid sequence is administered to immunologically compromised individuals for the purpose of stimulating systemic defence.

Serum from the immunised animal is collected and treated according to known procedures. If serum containing polyclonal antibodies to an epitope obtainable from a polypeptide as described here contains antibodies to other antigens, the polyclonal antibodies can be purified by immunoaffinity chromatography. Techniques for producing and processing polyclonal antisera are known in the art. In order that such antibodies may be made, we also provide amino acid sequences or fragments thereof haptenised to another amino acid sequence for use as immunogens in animals or humans.

Monoclonal antibodies directed against epitopes obtainable from a polypeptide or peptide as described here can also be readily produced by one skilled in the art. The general methodology for making monoclonal antibodies by hybridomas is well known. Immortal antibody-producing cell lines can be created by cell fusion, and also by other techniques such as direct transformation of B lymphocytes with oncogenic DNA, or transfection with Epstein-Barr virus. Panels of monoclonal antibodies produced against orbit epitopes can be screened for various properties; i.e., for isotype and epitope affinity.

Monoclonal antibodies may be prepared using any technique which provides for the production of antibody molecules by continuous cell lines in culture. These include, but are not limited to, the hybridoma technique originally described by Koehler and Milstein (1975 Nature 256:495-497), the trioma technique, the human B-cell hybridoma technique (Kosbor *et al* (1983) Immunol Today 4:72; Cote *et al* (1983) Proc Natl Acad Sci 80:2026-2030) and the EBV-hybridoma technique (Cole et al., *Monoclonal Antibodies and Cancer Therapy*, pp. 77-96, Alan R. Liss, Inc., 1985).

In addition, techniques developed for the production of "chimeric antibodies", the splicing of mouse antibody genes to human antibody genes to obtain a molecule with appropriate antigen specificity and biological activity can be used (Morrison *et al* (1984)

Proc Natl Acad Sci 81:6851-6855; Neuberger *et al* (1984) Nature 312:604-608; Takeda *et al* (1985) Nature 314:452-454). Alternatively, techniques described for the production of single chain antibodies (US Patent No. 4,946,779) can be adapted to produce the substance specific single chain antibodies.

Antibodies, both monoclonal and polyclonal, which are directed against epitopes obtainable from a polypeptide or peptide as described here are particularly useful in diagnosis, and those which are neutralising are useful in passive immunotherapy. Monoclonal antibodies, in particular, may be used to raise anti-idiotype antibodies. Anti-idiotype antibodies are immunoglobulins which carry an "internal image" of the substance and/or agent against which protection is desired. Techniques for raising anti-idiotype antibodies are known in the art. These anti-idiotype antibodies may also be useful in therapy.

Antibodies may also be produced by inducing *in vivo* production in the lymphocyte population or by screening recombinant immunoglobulin libraries or panels of highly specific binding reagents as disclosed in Orlandi *et al* (1989, Proc Natl Acad Sci 86: 3833-3837), and Winter G and Milstein C (1991; Nature 349:293-299).

Antibody fragments which contain specific binding sites for the polypeptide or peptide may also be generated. For example, such fragments include, but are not limited to, the $F(ab')_2$ fragments which can be produced by pepsin digestion of the antibody molecule and the Fab fragments which can be generated by reducing the disulfide bridges of the $F(ab')_2$ fragments. Alternatively, Fab expression libraries may be constructed to allow rapid and easy identification of monoclonal Fab fragments with the desired specificity (Huse WD *et al* (1989) Science 256:1275-128 1).

Techniques for the production of single chain antibodies (U.S. Pat. No. 4,946,778) can also be adapted to produce single chain antibodies to polypeptides as described here. Also, transgenic mice, or other organisms including other mammals, may be used to express humanized antibodies.

The above-described antibodies may be employed to isolate or to identify clones expressing the polypeptide or to purify the polypeptides by affinity chromatography.

Antibodies against Conrad GPCR polypeptides may also be employed to treat infections such as bacterial, fungal, protozoan and viral infections, particularly infections caused by HIV-1 or HIV-2; pain; cancers; diabetes, obesity; anorexia; bulimia; asthma; Parkinson's disease; thrombosis; acute heart failure; hypotension; hypertension; erectile dysfunction; urinary retention; metabolic bone diseases such as osteoporisis and osteo petrosis; angina pectoris; myocardial infarction; ulcers; asthma; allergies; rheumatoid arthritis; inflammatory bowel disease; irritable bowel syndrome benign prostatic hypertrophy; and psychotic and neurological disorders, including anxiety, schizophrenia, manic depression, delirium, dementia, severe mental retardation and dyskinesias, such as Huntington's disease or Gilles dela Tourett's syndrome.

In a particular embodiment, antibodies against Conrad GPCR polypeptides are employed to treat any of the following diseases: long QT syndrome-4 with sinus bradycardia disease, mental health wellness-2 disease, psoriasis or susceptibility to psoriasis, dentin dysplasia, type II disease and neutropenia, neonatal alloimmune disease.

DIAGNOSTIC ASSAYS

We further describe the use of Conrad GPCR polynucleotides and polypeptides (as well as homologues, variants and derivatives thereof) for use in diagnosis as diagnostic reagents or in genetic analysis. Nucleic acids complementary to or capable of hybridising to Conrad GPCR nucleic acids (including homologues, variants and derivatives), as well as antibodies against Conrad polypeptides are also useful in such assays.

Detection of a mutated form of the Conrad GPCR gene associated with a dysfunction will provide a diagnostic tool that can add to or define a diagnosis of a disease or susceptibility to a disease which results from under-expression, over-expression or altered expression of Conrad GPCR. Individuals carrying mutations in the Conrad GPCR

gene (including control sequences) may be detected at the DNA level by a variety of techniques.

For example, DNA may be isolated from a patient and the DNA polymorphism pattern of Conrad determined. The identified pattern is compared to controls of patients known to be suffering from a disease associated with over-, under- or abnormal expression of Conrad. Patients expressing a genetic polymorphism pattern associated with Conrad associated disease may then be identified. Genetic analysis of the Conrad GPCR gene may be conducted by any technique known in the art. For example, individuals may be screened by determining DNA sequence of a Conrad allele, by RFLP or SNP analysis, etc. Patients may be identified as having a genetic predisposition for a disease associated with the over-, under-, or abnormal expression of Conrad by detecting the presence of a DNA polymorphism in the gene sequence for Conrad or any sequence controlling its expression.

Patients so identified can then be treated to prevent the occurrence of Conrad associated disease, or more aggressively in the early stages of Conrad associated disease to prevent the further occurrence or development of the disease. Conrad associated diseases include infections such as bacterial, fungal, protozoan and viral infections, particularly infections caused by HIV-1 or HIV-2; pain; cancers; diabetes, obesity; anorexia; bulimia; asthma; Parkinson's disease; thrombosis; acute heart failure; hypotension; hypertension; erectile dysfunction; urinary retention; metabolic bone diseases such as osteoporisis and osteo petrosis; angina pectoris; myocardial infarction; ulcers; asthma; allergies; rheumatoid arthritis; inflammatory bowel disease; irritable bowel syndrome benign prostatic hypertrophy; and psychotic and neurological disorders, including anxiety, schizophrenia, manic depression, delirium, dementia, severe mental retardation and dyskinesias, such as Huntington's disease or Gilles dela Tourett's syndrome.

In a preferred embodiment, Conrad associated diseases comprise any one of long QT syndrome-4 with sinus bradycardia disease, mental health wellness-2 disease, psoriasis or susceptibility to psoriasis, dentin dysplasia, type II disease and neutropenia, neonatal alloimmune disease.

We further disclose a kit for the identification of a patient's genetic polymorphism pattern associated with Conrad associated disease. The kit includes DNA sample collecting means and means for determining a genetic polymorphism pattern, which is then compared to control samples to determine a patient's susceptibility to Conrad associated disease. Kits for diagnosis of a Conrad associated disease comprising Conrad polypeptide and/or an antibody against such a polypeptide (or fragment of it) are also provided.

Nucleic acids for diagnosis may be obtained from a subject's cells, such as from blood, urine, saliva, tissue biopsy or autopsy material. In a preferred embodiment, the DNA is obtained from blood cells obtained from a finger prick of the patient with the blood collected on absorbent paper. In a further preferred embodiment, the blood will be collected on an AmpliCard.TM. (University of Sheffield, Department of Medicine and Pharmacology, Royal Hallamshire Hospital, Sheffield, England S10 2JF).

The DNA may be used directly for detection or may be amplified enzymatically by using PCR or other amplification techniques prior to analysis. Oligonucleotide DNA primers that target the specific polymorphic DNA region within the genes of interest may be prepared so that in the PCR reaction amplification of the target sequences is achieved. RNA or cDNA may also be used as templates in similar fashion. The amplified DNA sequences from the template DNA may then be analyzed using restriction enzymes to determine the genetic polymorphisms present in the amplified sequences and thereby provide a genetic polymorphism profile of the patient. Restriction fragments lengths may be identified by gel analysis. Alternatively, or in conjunction, techniques such as SNP (single nucleotide polymorphisms) analysis may be employed.

Deletions and insertions can be detected by a change in size of the amplified product in comparison to the normal genotype. Point mutations can be identified by hybridizing amplified DNA to labeled Conrad GPCR nucleotide sequences. Perfectly matched sequences can be distinguished from mismatched duplexes by RNase digestion or by differences in melting temperatures. DNA sequence differences may also be detected

by alterations in electrophoretic mobility of DNA fragments in gels, with or without denaturing agents, or by direct DNA sequencing. See, eg., Myers et al, Science (1985)230:1242. Sequence changes at specific locations may also be revealed by nuclease protection assays, such as RNase and S1protection or the chemical cleavage method. See Cotton et al., *Proc Natl Acad Sci USA* (1985) 85: 4397-4401. In another embodiment, an array of oligonucleotides probes comprising the Conrad GPCR nucleotide sequence or fragments thereof can be constructed to conduct efficient screening of e.g., genetic mutations. Array technology methods are well known and have general applicability and can be used to address a variety of questions in molecular genetics including gene expression, genetic linkage, and genetic variability. (See for example: M.Chee et al., *Science*, Vol 274, pp 610-613 (1996)).

Single strand conformation polymorphism (SSCP) may be used to detect differences in electrophoretic mobility between mutant and wild type nucleic acids (Orita et al. (1989) *Proc Natl. Acad. Sci USA*: 86:2766, see also Cotton (1993) *Mutat Res* 285:125-144; and Hayashi (1992) *Genet Anal Tech Appl* 9:73-79). Single-stranded DNA fragments of sample and control Conrad nucleic acids may be denatured and allowed to renature. The secondary structure of single-stranded nucleic acids varies according to sequence, the resulting alteration in electrophoretic mobility enables the detection of even a single base change. The DNA fragments may be labelled or detected with labelled probes. The sensitivity of the assay may be enhanced by using RNA (rather than DNA), in which the secondary structure is more sensitive to a change in sequence. In a preferred embodiment, the subject method utilizes heteroduplex analysis to separate double stranded heteroduplex molecules on the basis of changes in electrophoretic mobility (Keen et al. (1991) *Trends Genet* 7:5).

The diagnostic assays offer a process for diagnosing or determining a susceptibility to infections such as infections such as bacterial, fungal, protozoan and viral infections, particularly infections caused by HIV-1 or HIV-2; pain; cancers; diabetes, obesity; anorexia; bulimia; asthma; Parkinson's disease; thrombosis; acute heart failure; hypotension; hypertension; erectile dysfunction; urinary retention; metabolic bone

diseases such as osteoporisis and osteo petrosis; angina pectoris; myocardial infarction; ulcers; asthma; allergies; rheumatoid arthritis; inflammatory bowel disease; irritable bowel syndrome benign prostatic hypertrophy; and psychotic and neurological disorders, including anxiety, schizophrenia, manic depression, delirium, dementia, severe mental retardation and dyskinesias, such as Huntington's disease or Gilles dela Tourett's syndrome through detection of mutation in the Conrad GPCR gene by the methods described.

In a particularly preferred embodiment, the diagnostic assays are used to diagnose or determine susceptibility to long QT syndrome-4 with sinus bradycardia disease, mental health wellness-2 disease, psoriasis or susceptibility to psoriasis, dentin dysplasia, type II disease or neutropenia, neonatal alloimmune disease.

The presence of Conrad GPCR polypeptides and nucleic acids may be detected in a sample. Thus, infections and diseases as listed above can be diagnosed by methods comprising determining from a sample derived from a subject an abnormally decreased or increased level of the Conrad GPCR polypeptide or Conrad GPCR mRNA. The sample may comprise a cell or tissue sample from an organism suffering or suspected to be suffering from a disease associated with increased, reduced or otherwise abnormal Conrad GPCR expression, including spatial or temporal changes in level or pattern of expression. The level or pattern of expression of Conrad in an organism suffering from or suspected to be suffering from such a disease may be usefully compared with the level or pattern of expression in a normal organism as a means of diagnosis of disease.

In general therefore, we disclose a method of detecting the presence of a nucleic acid comprising a Conrad GPCR nucleic acid in a sample, by contacting the sample with at least one nucleic acid probe which is specific for said nucleic acid and monitoring said sample for the presence of the nucleic acid. For example, the nucleic acid probe may specifically bind to the Conrad GPCR nucleic acid, or a portion of it, and binding between the two detected; the presence of the complex itself may also be detected. Furthermore, we describe a method of detecting the presence of a Conrad GPCR polypeptide by contacting a cell sample with an antibody capable of binding the polypeptide and monitoring said

sample for the presence of the polypeptide. This may conveniently be achieved by monitoring the presence of a complex formed between the antibody and the polypeptide, or monitoring the binding between the polypeptide and the antibody. Methods of detecting binding between two entities are known in the art, and include FRET (fluorescence resonance energy transfer), surface plasmon resonance, etc.

Decreased or increased expression can be measured at the RNA level using any of the methods well known in the art for the quantitation of polynucleotides, such as, for example, PCR, RT-PCR, RNase protection, Northern blotting and other hybridization methods. Assay techniques that can be used to determine levels of a protein, such as a Conrad GPCR, in a sample derived from a host are well-known to those of skill in the art. Such assay methods include radioimmunoassays, competitive-binding assays, Western Blot analysis and ELISA assays.

This document also relates to a diagnostic kit for a disease or susceptibility to a disease (including an infection), for example, infections such as bacterial, fungal, protozoan and viral infections, particularly infections caused by HIV-1 or HIV-2; pain; cancers; diabetes, obesity; anorexia; bulimia; asthma; Parkinson's disease; thrombosis; acute heart failure; hypotension; hypertension; erectile dysfunction; urinary retention; metabolic bone diseases such as osteoporisis and osteo petrosis; angina pectoris; myocardial infarction; ulcers; asthma; allergies; rheumatoid arthritis; inflammatory bowel disease; irritable bowel syndrome benign prostatic hypertrophy; and psychotic and neurological disorders, including anxiety, schizophrenia, manic depression, delirium, dementia, severe mental retardation and dyskinesias, such as Huntington's disease or Gilles dela Tourett's syndrome.

A particularly preferred diagnostic kit is used to detect or diagnoise disease or susceptibility to any of the following: long QT syndrome-4 with sinus bradycardia disease, mental health wellness-2 disease, psoriasis or susceptibility to psoriasis, dentin dysplasia, type II disease and neutropenia, neonatal alloimmune disease.

The diagnostic kit comprises a Conrad GPCR polynucleotide or a fragment thereof; a complementary nucleotide sequence; a Conrad GPCR polypeptide or a fragment thereof, or an antibody to a Conrad GPCR polypeptide.

CHROMOSOME ASSAYS

The nucleotide sequences as described here are also valuable for chromosome identification. The sequence is specifically targeted to and can hybridize with a particular location on an individual human chromosome. As described above, human Conrad GPCR is found to map to Homo sapiens chromosome 4q26.

The mapping of relevant sequences to chromosomes is an important first step in correlating those sequences with gene associated disease. Once a sequence has been mapped to a precise chromosomal location, the physical position of the sequence on the chromosome can be correlated with genetic map data. Such data are found, for example, in V. McKusick, Mendelian heritance in Man (available on line through Johns Hopkins University Welch Medical Library). The relationship between genes and diseases that have been mapped to the same chromosomal region are then identified through linkage analysis (coinheritance of physically adjacent genes).

The differences in the cDNA or genomic sequence between affected and unaffected individuals can also be determined. If a mutation is observed in some or all of the affected individuals but not in any normal individuals, then the mutation is likely to be the causative agent of the disease.

PROPHYLACTIC AND THERAPEUTIC METHODS

We provide methods of treating an abnormal conditions related to both an excess of and insufficient amounts of Conrad GPCR activity.

If the activity of Conrad GPCR is in excess, several approaches are available. One approach comprises administering to a subject an inhibitor compound (antagonist) as hereinabove described along with a pharmaceutically acceptable carrier in an amount effective to inhibit activation by blocking binding of ligands to the Conrad GPCR, or by inhibiting a second signal, and thereby alleviating the abnormal condition.

In another approach, soluble forms of Conrad GPCR polypeptides still capable of binding the ligand in competition with endogenous Conrad GPCR may be administered. Typical embodiments of such competitors comprise fragments of the Conrad GPCR polypeptide.

In still another approach, expression of the gene encoding endogenous Conrad GPCR can be inhibited using expression blocking techniques. Known such techniques involve the use of antisense sequences, either internally generated or separately administered. See, for example, O'Connor, *J Neurochem* (1991) 56:560 in Oligodeoxvnucleotides as Antisense Inhibitors of Gene Expression, CRC Press, Boca Raton, Fla. (1988). Alternatively, oligonucleotides which form triple helices with the gene can be supplied. See, for example, Lee et al., *Nucleic Acids Res* (1979) 6:3073; Cooney et al., *Science* (1988) 241:456; Dervan et al., *Science* (1991) 251:1360. These oligomers can be administered per se or the relevant oligomers can be expressed in vivo.

For treating abnormal conditions related to an under-expression of Conrad GPCR and its activity, several approaches are also available. One approach comprises administering to a subject a therapeutically effective amount of a compound which activates Conrad GPCR, i.e., an agonist as described above, in combination with a pharmaceutically acceptable carrier, to thereby alleviate the abnormal condition. Alternatively, gene therapy may be employed to effect the endogenous production of Conrad GPCR by the relevant cells in the subject. For example, a polynucleotide as described here may be engineered for expression in a replication defective retroviral vector, as discussed above. The retroviral expression construct may then be isolated and introduced into a packaging cell transduced with a retroviral plasmid vector containing

RNA encoding a polypeptide as described here such that the packaging cell now produces infectious viral particles containing the gene of interest. These producer cells may be administered to a subject for engineering cells in vivo and expression of the polypeptide in vivo. For overview of gene therapy, see Chapter 20, Gene Therapy and other Molecular Genetic-based Therapeutic Approaches, (and references cited therein) in Human Molecular Genetics, T Strachan and A P Read, BIOS Scientific Publishers Ltd (1996).

FORMULATION AND ADMINISTRATION

Peptides, such as the soluble form of Conrad GPCR polypeptides, and agonists and antagonist peptides or small molecules, may be formulated in combination with a suitable pharmaceutical carrier. Such formulations comprise a therapeutically effective amount of the polypeptide or compound, and a pharmaceutically acceptable carrier or excipient. Such carriers include but are not limited to, saline, buffered saline, dextrose, water, glycerol, ethanol, and combinations thereof. Formulation should suit the mode of administration, and is well within the skill of the art. We further disclose pharmaceutical packs and kits comprising one or more containers filled with one or more of the ingredients of the aforementioned compositions.

Polypeptides and other compounds as described here may be employed alone or in conjunction with other compounds, such as therapeutic compounds.

Preferred forms of systemic administration of the pharmaceutical compositions include injection, typically by intravenous injection. Other injection routes, such as subcutaneous, intramuscular, or intraperitoneal, can be used. Alternative means for systemic administration include transmucosal and transdermal administration using penetrants such as bile salts or fusidic acids or other detergents. In addition, if properly formulated in enteric or encapsulated formulations, oral administration may also be possible. Administration of these compounds may also be topical and/or localize, in the form of salves, pastes, gels and the like.

The dosage range required depends on the choice of peptide, the route of administration, the nature of the formulation, the nature of the subject's condition, and the judgment of the attending practitioner. Suitable dosages, however, are in the range of 0.1-100 μg/kg of subject. Wide variations in the needed dosage, however, are to be expected in view of the variety of compounds available and the differing efficiencies of various routes of administration. For example, oral administration would be expected to require higher dosages than administration by intravenous injection. Variations in these dosage levels can be adjusted using standard empirical routines for optimization, as is well understood in the art.

Polypeptides used in treatment can also be generated endogenously in the subject, in treatment modalities often referred to as "gene therapy" as described above. Thus, for example, cells from a subject may be engineered with a polynucleotide, such as a DNA or RNA, to encode a polypeptide ex vivo, and for example, by the use of a retroviral plasmid vector. The cells are then introduced into the subject.

PHARMACEUTICAL COMPOSITIONS

We also provide a pharmaceutical composition comprising administering a therapeutically effective amount of the polypeptide, polynucleotide, peptide, vector or antibody as described here and optionally a pharmaceutically acceptable carrier, diluent or excipients (including combinations thereof).

The pharmaceutical compositions may be for human or animal usage in human and veterinary medicine and will typically comprise any one or more of a pharmaceutically acceptable diluent, carrier, or excipient. Acceptable carriers or diluents for therapeutic use are well known in the pharmaceutical art, and are described, for example, in Remington's Pharmaceutical Sciences, Mack Publishing Co. (A. R. Gennaro edit. 1985). The choice of pharmaceutical carrier, excipient or diluent can be selected with regard to the intended route of administration and standard pharmaceutical practice. The pharmaceutical

compositions may comprise as - or in addition to - the carrier, excipient or diluent any suitable binder(s), lubricant(s), suspending agent(s), coating agent(s), solubilising agent(s).

Preservatives, stabilizers, dyes and even flavoring agents may be provided in the pharmaceutical composition. Examples of preservatives include sodium benzoate, sorbic acid and esters of p-hydroxybenzoic acid. Antioxidants and suspending agents may be also used.

There may be different composition/formulation requirements dependent on the different delivery systems. By way of example, the pharmaceutical composition may be formulated to be delivered using a mini-pump or by a mucosal route, for example, as a nasal spray or aerosol for inhalation or ingestable solution, or parenterally in which the composition is formulated by an injectable form, for delivery, by, for example, an intravenous, intramuscular or subcutaneous route. Alternatively, the formulation may be designed to be delivered by both routes.

Where the agent is to be delivered mucosally through the gastrointestinal mucosa, it should be able to remain stable during transit though the gastrointestinal tract; for example, it should be resistant to proteolytic degradation, stable at acid pH and resistant to the detergent effects of bile.

Where appropriate, the pharmaceutical compositions can be administered by inhalation, in the form of a suppository or pessary, topically in the form of a lotion, solution, cream, ointment or dusting powder, by use of a skin patch, orally in the form of tablets containing excipients such as starch or lactose, or in capsules or ovules either alone or in admixture with excipients, or in the form of elixirs, solutions or suspensions containing flavouring or colouring agents, or they can be injected parenterally, for example intravenously, intramuscularly or subcutaneously. For parenteral administration, the compositions may be best used in the form of a sterile aqueous solution which may contain other substances, for example enough salts or monosaccharides to make the solution isotonic with blood. For buccal or sublingual administration the compositions

may be administered in the form of tablets or lozenges which can be formulated in a conventional manner.

VACCINES

Another embodiment relates to a method for inducing an immunological response in a mammal which comprises inoculating the mammal with the Conrad GPCR polypeptide, or a fragment thereof, adequate to produce antibody and/or T cell immune response to protect said animal from infections such as bacterial, fungal, protozoan and viral infections, particularly infections caused by HIV-1 or HIV-2; pain; cancers; diabetes, obesity; anorexia; bulimia; asthma; Parkinson's disease; thrombosis; acute heart failure; hypotension; hypertension; erectile dysfunction; urinary retention; metabolic bone diseases such as osteoporisis and osteo petrosis; angina pectoris; myocardial infarction; ulcers; asthma; allergies; rheumatoid arthritis; inflammatory bowel disease; irritable bowel syndrome benign prostatic hypertrophy; and psychotic and neurological disorders, including anxiety, schizophrenia, manic depression, delirium, dementia, severe mental retardation and dyskinesias, such as Huntington's disease or Gilles dela Tourett's syndrome, among others.

The induced immune response may also be employed to protect the animal from other diseases such as long QT syndrome-4 with sinus bradycardia disease, mental health wellness-2 disease, psoriasis or susceptibility to psoriasis, dentin dysplasia, type II disease and neutropenia, neonatal alloimmune disease.

Yet another embodiment relates to a method of inducing immunological response in a mammal which comprises delivering a Conrad GPCR polypeptide via a vector directing expression of a Conrad GPCR polynucleotide in vivo in order to induce such an immunological response to produce antibody to protect said animal from diseases.

A further embodiment relates to an immunological/vaccine formulation (composition) which, when introduced into a mammalian host, induces an immunological

response in that mammal to a Conrad GPCR polypeptide wherein the composition comprises a Conrad GPCR polypeptide or Conrad GPCR gene. The vaccine formulation may further comprise a suitable carrier.

Since the Conrad GPCR polypeptide may be broken down in the stomach, it is preferably administered parenterally (including subcutaneous, intramuscular, intravenous, intradermal etc. injection). Formulations suitable for parenteral administration include aqueous and non-aqueous sterile injection solutions which may contain anti-oxidants, buffers, bacteriostats and solutes which render the formulation instonic with the blood of the recipient; and aqueous and non-aqueous sterile suspensions which may include suspending agents or thickening agents. The formulations may be presented in unit-dose or multi-dose containers, for example, sealed ampoules and vials and may be stored in a freeze-dried condition requiring only the addition of the sterile liquid carrier immediately prior to use. The vaccine formulation may also include adjuvant systems for enhancing the immunogenicity of the formulation, such as oil-in water systems and other systems known in the art. The dosage will depend on the specific activity of the vaccine and can be readily determined by routine experimentation.

Vaccines may be prepared from one or more polypeptides or peptides as described here.

The preparation of vaccines which contain an immunogenic polypeptide(s) or peptide(s) as active ingredient(s), is known to one skilled in the art. Typically, such vaccines are prepared as injectables, either as liquid solutions or suspensions; solid forms suitable for solution in, or suspension in, liquid prior to injection may also be prepared. The preparation may also be emulsified, or the protein encapsulated in liposomes. The active immunogenic ingredients are often mixed with excipients which are pharmaceutically acceptable and compatible with the active ingredient. Suitable excipients are, for example, water, saline, dextrose, glycerol, ethanol, or the like and combinations thereof.

In addition, if desired, the vaccine may contain minor amounts of auxiliary substances such as wetting or emulsifying agents, pH buffering agents, and/or adjuvants which enhance the effectiveness of the vaccine. Examples of adjuvants which may be effective include but are not limited to: aluminum hydroxide, N-acetyl-muramyl-L-threonyl-D-isoglutamine (thr-MDP), N-acetyl-nor-muramyl-L-alanyl-D-isoglutamine (CGP 11637, referred to as nor-MDP), N-acetylmuramyl-L-alanyl-D-isoglutaminyl-L-alanine-2-(1'-2'-dipalmitoyl-sn-glycero-3-hydroxyphosphoryloxy)-ethylamine (CGP 19835A, referred to as MTP-PE), and RIBI, which contains three components extracted from bacteria, monophosphoryl lipid A, trehalose dimycolate and cell wall skeleton (MPL+TDM+CWS) in a 2% squalene/Tween 80 emulsion.

Further examples of adjuvants and other agents include aluminum hydroxide, aluminum phosphate, aluminum potassium sulfate (alum), beryllium sulfate, silica, kaolin, carbon, water-in-oil emulsions, oil-in-water emulsions, muramyl dipeptide, bacterial endotoxin, lipid X, *Corynebacterium parvum* (*Propionobacterium acnes*), *Bordetella pertussis*, polyribonucleotides, sodium alginate, lanolin, lysolecithin, vitamin A, saponin, liposomes, levamisole, DEAE-dextran, blocked copolymers or other synthetic adjuvants. Such adjuvants are available commercially from various sources, for example, Merck Adjuvant 65 (Merck and Company, Inc., Rahway, N.J.) or Freund's Incomplete Adjuvant and Complete Adjuvant (Difco Laboratories, Detroit, Michigan).

Typically, adjuvants such as Amphigen (oil-in-water), Alhydrogel (aluminum hydroxide), or a mixture of Amphigen and Alhydrogel are used. Only aluminum hydroxide is approved for human use.

The proportion of immunogen and adjuvant can be varied over a broad range so long as both are present in effective amounts. For example, aluminum hydroxide can be present in an amount of about 0.5% of the vaccine mixture (Al_2O_3 basis). Conveniently, the vaccines are formulated to contain a final concentration of immunogen in the range of from 0.2 to 200 μg/ml, preferably 5 to 50 μg/ml, most preferably 15 μg/ml.

After formulation, the vaccine may be incorporated into a sterile container which is then sealed and stored at a low temperature, for example 4°C, or it may be freeze-dried. Lyophilisation permits long-term storage in a stabilised form.

The vaccines are conventionally administered parenterally, by injection, for example, either subcutaneously or intramuscularly. Additional formulations which are suitable for other modes of administration include suppositories and, in some cases, oral formulations. For suppositories, traditional binders and carriers may include, for example, polyalkylene glycols or triglycerides; such suppositories may be formed from mixtures containing the active ingredient in the range of 0.5% to 10%, preferably 1% to 2%. Oral formulations include such normally employed excipients as, for example, pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium saccharine, cellulose, magnesium carbonate, and the like. These compositions take the form of solutions, suspensions, tablets, pills, capsules, sustained release formulations or powders and contain 10% to 95% of active ingredient, preferably 25% to 70%. Where the vaccine composition is lyophilised, the lyophilised material may be reconstituted prior to administration, e.g. as a suspension. Reconstitution is preferably effected in buffer

Capsules, tablets and pills for oral administration to a patient may be provided with an enteric coating comprising, for example, Eudragit "S", Eudragit "L", cellulose acetate, cellulose acetate phthalate or hydroxypropylmethyl cellulose.

The polypeptides as described here may be formulated into the vaccine as neutral or salt forms. Pharmaceutically acceptable salts include the acid addition salts (formed with free amino groups of the peptide) and which are formed with inorganic acids such as, for example, hydrochloric or phosphoric acids, or such organic acids such as acetic, oxalic, tartaric and maleic. Salts formed with the free carboxyl groups may also be derived from inorganic bases such as, for example, sodium, potassium, ammonium, calcium, or ferric hydroxides, and such organic bases as isopropylamine, trimethylamine, 2-ethylamino ethanol, histidine and procaine.

ADMINISTRATION

Typically, a physician will determine the actual dosage which will be most suitable for an individual subject and it will vary with the age, weight and response of the particular patient. The dosages below are exemplary of the average case. There can, of course, be individual instances where higher or lower dosage ranges are merited.

The pharmaceutical and vaccine compositions as described here may be administered by direct injection. The composition may be formulated for parenteral, mucosal, intramuscular, intravenous, subcutaneous, intraocular or transdermal administration. Typically, each protein may be administered at a dose of from 0.01 to 30 mg/kg body weight, preferably from 0.1 to 10 mg/kg, more preferably from 0.1 to 1 mg/kg body weight.

The term "administered" includes delivery by viral or non-viral techniques. Viral delivery mechanisms include but are not limited to adenoviral vectors, adeno-associated viral (AAV) vectos, herpes viral vectors, retroviral vectors, lentiviral vectors, and baculoviral vectors. Non-viral delivery mechanisms include lipid mediated transfection, liposomes, immunoliposomes, lipofectin, cationic facial amphiphiles (CFAs) and combinations thereof. The routes for such delivery mechanisms include but are not limited to mucosal, nasal, oral, parenteral, gastrointestinal, topical, or sublingual routes.

The term "administered" includes but is not limited to delivery by a mucosal route, for example, as a nasal spray or aerosol for inhalation or as an ingestable solution; a parenteral route where delivery is by an injectable form, such as, for example, an intravenous, intramuscular or subcutaneous route.

The term "co-administered" means that the site and time of administration of each of for example, the polypeptide as described here and an additional entity such as adjuvant are such that the necessary modulation of the immune system is achieved. Thus, whilst the polypeptide and the adjuvant may be administered at the same moment in time and at the

same site, there may be advantages in administering the polypeptide at a different time and to a different site from the adjuvant. The polypeptide and adjuvant may even be delivered in the same delivery vehicle - and the polypeptide and the antigen may be coupled and/or uncoupled and/or genetically coupled and/or uncoupled.

The polypeptide, polynucleotide, peptide, nucleotide, antibody and optionally an adjuvant may be administered separately or co-administered to the host subject as a single dose or in multiple doses.

The vaccine composition and pharmaceutical compositions may be administered by a number of different routes such as injection (which includes parenteral, subcutaneous and intramuscular injection) intranasal, mucosal, oral, intra-vaginal, urethral or ocular administration.

The vaccines and pharmaceutical compositions may be conventionally administered parenterally, by injection, for example, either subcutaneously or intramuscularly. Additional formulations which are suitable for other modes of administration include suppositories and, in some cases, oral formulations. For suppositories, traditional binders and carriers may include, for example, polyalkylene glycols or triglycerides; such suppositories may be formed from mixtures containing the active ingredient in the range of 0.5% to 10%, may be 1% to 2%. Oral formulations include such normally employed excipients as, for example, pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium saccharine, cellulose, magnesium carbonate, and the like. These compositions take the form of solutions, suspensions, tablets, pills, capsules, sustained release formulations or powders and contain 10% to 95% of active ingredient, preferably 25% to 70%. Where the vaccine composition is lyophilised, the lyophilised material may be reconstituted prior to administration, e.g. as a suspension. Reconstitution is preferably effected in buffer.

EXAMPLES

Example 1. Transgenic CONRAD Knock-Out Mouse

Construction of CONRAD Gene Targeting Vector

The murine CONRAD gene is identified and consists of six coding exons. A genomic contig comprising 31kb of uninterrupted sequence and containing the first coding exon is obtained. This contig provided sufficient flanking sequence information to enable the design of homologous arms to clone into the targeting vector (the structure of the targeting vector used, including the relevant restriction sites, is shown in Figure 5).

The murine CONRAD gene has six coding exons. The targeting strategy is designed to remove part of the first coding exon, including the start of the 7tm coding domain. A 1.7 kb 5' homologous arm and a 3.2 kb 3' homologous arm flanking the region to be deleted are amplified by PCR and the fragments are cloned into the targeting vector. The 5' end of each oligonucleotide primer used to amplify the arms is synthesized to contain a different recognition site for a rare-cutting restriction enzyme, compatible with the cloning sites of the vector polylinkers and absent from the arms themselves. In the case of CONRAD, the primers are designed as listed in the sequence table below, with 5' arm cloning enzymes of NotI/SpeI and 3' arm cloning enzymes of AscI/FseI.

In addition to the arm primer pairs (5'armF/5'armR and 3'armF/3'armR2), further primers specific to the CONRAD locus are designed for the following purposes: 5' and 3' probe primer pairs (5'prF/5'prR and 3'prF2/3'prR) to amplify two short 150-300bp fragments of non-repetitive genomic DNA external to and extending beyond each arm, to allow Southern analysis of the targeted locus, in isolated putative targeted clones; a mouse genotyping primer pair (hetF and hetR) which allows differentiation between wild-type, heterozygote and homozygous mice, when used in a multiplex PCR with a vector-specific primer, in this case, Asc306; and lastly, a target screening primer (5'scr) which anneals upstream of the end of the 5' arm region, and which produces a target event specific 1.8kb amplimer when paired with a primer specific to the 5' end of the vector (DR2). This

amplimer can only be derived from template DNA from cells where the desired genomic alteration has occurred and allows the identification of correctly targeted cells from the background of clones containing randomly integrated copies of the vector. The location of these primers and the genomic structure of the CONRAD locus used in the targeting strategy is shown in SEQ ID NO: 18.

musConrad 5'prF DR2	CGAAATATGAAGGAGTAAGGAGAGCAG
musConrad 5'prR	GATTGCGTTGACTTTGCATTAAATTCTG
musConrad 5'scr DR2	CTACAGAATTTAATGCAAAGTCAACGCAATC
musConrad 5'armF Not	tttgcggccgCAACATTTAAAATATATTCTGGGGCTG
musConrad 5'armR Spe	aaaactagtGCGATGAATGAACTGTTCCCGAGTCAG
musConrad 3'armF Asc	aaaggcgcgccAGACAGCGATTACGCGTGCACACTCAC
musConrad 3'armR2 Fse	tttggccggcCTTTCCAGGCATCAATTGTTGCTGTTG
musConrad 3'prF.2	GAAATTTGATCAGATTCACCCTTCATATCC
musConrad 3'prR	AAGATTGTTATGTGCAGGCTGGAGGTG
musConrad hetF	GGAGCACTCATTTTTGCCCTGGCGCTC
musConrad hetR a306	TCCGTGAGTGTGCACGCGTAATCGCTG
Asc306	AATGGCCGCTTTTCTGGATTCATCGAC
DR2	ATCATGGCCCTACCATGCGCTAAACAC

Table 1. CONRAD Primer Sequences

The position of the homology arms is chosen to functionally disrupt the CONRAD gene by deleting a region just downstream of the endogenous ATG and including several of the seven transmembrane spanning regions present in the first coding exon. A targeting vector is prepared where the deleted CONRAD sequence is replaced with non-homologous sequences composed of an endogenous gene expression reporter (a frame independent lacZ gene) upstream of a selection cassette composed of a promoted neomycin phosphotransferase (neo) gene arranged in the same orientation as the CONRAD gene.

Once the 5' and 3' homology arms had been cloned into the targeting vector pTK5IBLMNL (see Figure 5), a large highly pure DNA preparation is made using standard molecular biology techniques. 20 μg of the freshly prepared endotoxin free DNA is restricted with another rare-cutting restriction enzyme PmeI, present at a unique site in the vector backbone between the ampicillin resistance gene and the bacterial origin of

replication. The linearized DNA is then precipitated and resuspended in 100 μl of Phosphate Buffered Saline, ready for electroporation.

24 hours following electroporation the transfected cells are cultured for 9 days in medium containing 200 μg/ml neomycin. Clones are picked into 96 well plates, replicated and expanded before being screened by PCR (using primers 5'scr and DR2, as described above) to identify clones in which homologous recombination had occurred between the endogenous CONRAD gene and the targeting construct. Positive clones can be identified at a rate of 1 to 5%. These clones are expanded to allow replicas to be frozen and sufficient high quality DNA to be prepared for Southern blot confirmation of the targeting event using the external 5' and 3' probes prepared as described above, all using standard procedures (Russ et al, Nature 2000 Mar 2;404(6773):95-92000). When Southern blots of DNA digested with diagnostic restriction enzymes are hybridized with an external probe, homologously targeted ES cell clones are verified by the presence of a mutant band as well an unaltered wild-type band. For instance, , BamHI digested DNA will give a 12kb wild-type band, with a 2kb targeted band using the 5' probe and a 9.5 kb band with the 3' probe; PvuII will give a 10kb wild-type band, with a 6.2kb targeted band using the 5' probe and a 6kb band with the 3' probe.

The structure of the genomic locus of mouse CONRAD before knock-out is depicted in Figure 3. The structure of the genomic locus of mouse CONRAD after knock-out is depicted in Figure 4. The sites for the enzymes relevant to the Southern verification have been annotated.

Generation of CONRAD GPCR Deficient Mice

C57BL/6 female and male mice are mated and blastocysts are isolated at 3.5 days of gestation. 10-12 cells from a chosen clone are injected per blastocyst and 7-8 blastocysts are implanted in the uterus of a pseudo-pregnant F1 female. A litter of chimeric pups are born containing several high-level (up to 100%) agouti males (the agouti coat colour indicates the contribution of cells descendent from the targeted clone).

These male chimeras are mated with female and MF1 and 129 mice, and germ-line transmission is determined by the agouti coat colour and by PCR genotyping respectively.

PCR Genotyping is carried out on lysed tail clips, using the primers hetF and hetR with a third, vector specific primer (Asc306). This multiplex PCR allows amplification from the wild-type locus (if present) from primers hetF and hetR giving a 220 bp band. The site for hetF is deleted in the knockout mice, so this amplification will fail from a targeted allele. However, the Asc306 primer will amplify a 336 bp band from the targeted locus, in combination with the hetR primer which anneals to a region just inside the 3' arm. Therefore, this multiplex PCR reveals the genotype of the litters as follows: wild-type samples will exhibit a single 220 bp band; heterozygous DNA samples yield two bands at 220 bp and 336 bp; and the homozygous samples will show only the target specific 336 bp band.

Example 2. Expression of Recombinant CONRAD Protein

Recombinant CONRAD is expressed and purified. Two systems are used for expression.

pTOPO-Echo Donor Based Construct

A polynucleotide having the sequence shown in SEQ ID NO: 13 (below) is obtained from the human CONRAD nucleic acid sequence (SEQ ID NO:7). The SEQ ID NO: 13 polynucleotide is amplified by PCR using the oligonucleotide primers ATGCAGGCGCTTAACATTACCCCG and TGCCCACTGTCTAAAGGAGAATTC. This is cloned into a pTOPO-Echo Donor vector module (Invitrogen pUniV5/His Cat# ET001-10). This is then recombined into a suitable expression vector according to the host/expression system to be used. Transfection of the resulting construct into a host strain and induction of expression (according to the manufacturer's instructions) yields a fusion protein having the sequence of SEQ ID NO: 14.

The fusion polypeptide SEQ ID NO: 14 contains a C terminal V5 tag (residues 438 to 451) and His tag (residues 452 to 457) to aid detection and purification.

pCDNA5-JE Based Construct

A polynucleotide having the sequence shown in SEQ ID NO: 15 is amplified by PCR using the oligonucleotide primers AAATAAAGCTTGCAATGCAGGCGCTTAACATTACC and TATAAAGGATCCTTAATGCCCACTGTCTAAAGGAG to incorporate new restriction sites, HinDIII and BamHI at the 5-prime and 3-prime ends respectively of Conrad. This is then digested and ligated into similarly digested pcDNA5-JE (Invitrogen Cat# - K6010-01 vector modified to remove BGH Poly-A).

The resulting construct is used for high level expression in CHO-K1 cells , and other mammalian cell lines, under the control of the *cmv* promoter to yield a native polypeptide SEQ ID No: 9.

A polynucleotide having the sequence shown in SEQ ID NO: 16 is amplified by PCR using the oligonucleotide primers AAATAAAGCTTGCAATGCAGGCGCTTAACATTACC and TATAAAGGATCCTTACTTATCGTCGTCATCCTTGTAATCATGCCCACTGTCTAAAGGAG to incorporate new restriction sites, HinDIII and BamHI at the 5-prime and 3-prime ends respectively of Conrad and to include a 3' fusion FLAG tag. This is then digested and ligated into similarly digested pcDNA5-JE (Invitrogen Cat# - K6010-01 vector modified to remove BGH Poly-A).

The resulting construct is used for high level expression in CHO-K1 cells, and other mammalian cell lines, under the control of the *cmv* promoter to yield a fusion polypeptide with C terminal FLAG tag (double underline, residues432-439) to aid detection and purification. The resultant expressed fusion polypeptide has a sequence shown in SEQ ID NO: 17.

Introduction of Construct into Cells

The expression vector is introduced to the cells by lipofection (using Fugene-6 from Roche, Cat# 1 814 433) among other similar methods.

Both transient and stable transfection of these cells is achieved. In transient expression the cells are transfected by lipofection using a large amount of vector that results in a short-lived fast expression of the protein. In a stable transfection, the vector, which includes a selectable marker for neomycin resistance becomes stably integrated into the genome of the host cell resulting in a long-lived cell line with a high expression level of Conrad.

Cells expressing recombinant CONRAD are used for assay development, antibody production, and other purposes as described.

Expression in Other Host Cells

The recombinant/fusion Topo clone containing SEQ ID NO: 13 is recombined into a pBAD-Thio-E fector (Invitrogen Cat# ET100-01) for high level bacterial expression under control of the *ara*BAD promoter, using a Cre/Lox mediated recombination system.

The recombinant/fusion Topo clone containing SEQ ID NO: 13 is recombined into a pBlueBac 4.5E (Invitrogen Cat# ET310-01), using a Cre/Lox mediated recombination system, for subsequent recombination into Baculovirus expression systems. Recombination into MaxBac (Invitrogen Cat# K875-02) for high-level expression in SF9 and other insect cell lines.

The recombinant/fusion Topo clone containing SEQ ID NO: 13 is recombined into pcDNA 3.1-E (Invitrogen Cat# ET400-01) , using a Cre/Lox mediated recombination system, for high level expression in CHO-K1 (Chinese Hamster Ovary) cells, and other mammalian cell lines, under the control of the *cmv* promoter.

The invention will now be further described by the following numbered paragraphs:

1. A Conrad GPCR polypeptide comprising the amino acid sequence shown in SEQ ID NO: 3, SEQ ID NO: 5, SEQ ID NO: 9, SEQ ID NO: 11, SEQ ID NO: 14 or SEQ ID NO: 17, or a homologue, variant or derivative thereof.

2. A nucleic acid encoding a polypeptide according to Paragraph 1.

3. A nucleic acid according to Paragraph 2, comprising the nucleic acid sequence shown in SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 4, SEQ ID NO: 6, SEQ ID NO: 7, SEQ ID NO: 8, SEQ ID NO: 10, SEQ ID NO: 12, SEQ ID NO: 13, SEQ ID NO: 15, SEQ ID NO: 16 or SEQ ID NO: 18, or a homologue, variant or derivative thereof.

4. A polypeptide comprising a fragment of a polypeptide according to Paragraph 1.

5. A polypeptide according to Paragraph 3 which comprises one or more regions which are homologous between a pair of sequences selected from one of SEQ ID NO: 3 and SEQ ID NO: 9, and one of SEQ ID NO: 5 and SEQ ID NO: 11, or which comprises one or more regions which are heterologous between the pair.

6. A nucleic acid encoding a polypeptide according to Paragraph 4 or 5.

7. A vector comprising a nucleic acid according to Paragraph 2, 3, or 6.

8. A host cell comprising a nucleic acid according to Paragraph 2, 3, or 6, or vector according to Paragraph 7.

9. A transgenic non-human animal comprising a nucleic acid according to Paragraph 2, 3 or 6, or a vector according to Paragraph 7.

10. A transgenic non-human animal according to Paragraph 9 which is a mouse.

11. Use of a polypeptide according to Paragraph 1, 4 or 5 in a method of identifying a compound which is capable of interacting specifically with a G protein coupled receptor.

12. Use of a transgenic non-human animal according to Paragraph 9 or 10 in a method of identifying a compound which is capable of interacting specifically with a G protein coupled receptor.

13. A method for identifying an antagonist of a Conrad GPCR, the method comprising contacting a cell which expresses Conrad receptor with a candidate compound and determining whether the level of cyclic AMP (cAMP) in the cell is lowered as a result of said contacting.

14. A method for identifying a compound capable of lowering the endogenous level of cyclic AMP in a cell which method comprises contacting a cell which expresses a Conrad GPCR with a candidate compound and determining whether the level of cyclic AMP (cAMP) in the cell is lowered as a result of said contacting.

15. A method of identifying a compound capable of binding to a Conrad GPCR polypeptide, the method comprising contacting a Conrad GPCR polypeptide with a candidate compound and determining whether the candidate compound binds to the Conrad GPCR po!ypeptide.

16. A compound identified by a method according to any of Paragraphs 11 to 15.

17. A compound capable of binding specifically to a polypeptide according to Paragraph 1, 4 or 5.

18. Use of a polypeptide according to Paragraph 1, 4 or 5, or part thereof or a nucleic acid according to Paragraph 2, 3 or 6, in a method for producing antibodies.

19. An antibody capable of binding specifically to a polypeptide according to Paragraph 1, 4 or 5, or part thereof or a polypeptide encoded by a nucleotide according to Paragraph 2, 3 or 6 or part thereof.

20. A pharmaceutical composition comprising any one or more of the following: a polypeptide according to Paragraph 1, 4 or 5, or part thereof; a nucleic acid according to Paragraph 2, 3 or 6, or part thereof; a vector according to Paragraph 7; a cell according to Paragraph 8; a compound according to Paragraph 16 or 17; and an antibody according to Paragraph 19, together with a pharmaceutically acceptable carrier or diluent.

21. A vaccine composition comprising any one or more of the following: a polypeptide according to Paragraph 1, 4 or 5, or part thereof; a nucleic acid according to Paragraph 2, 3 or 6, or part thereof; a vector according to Paragraph 7; a cell according to Paragraph 8; a compound according to Paragraph 16 or 17; and an antibody according to Paragraph 19.

22. A diagnostic kit for a disease or susceptibility to a disease comprising any one or more of the following: a polypeptide according to Paragraph 1, 4 or 5, or part thereof; a nucleic acid according to Paragraph 2, 3 or 6, or part thereof; a vector according to Paragraph 7; a cell according to Paragraph 8; a compound according to Paragraph 16 or 17; and an antibody according to Paragraph 19.

23. A method of treating a patient suffering from a disease associated with enhanced activity of a Conrad GPCR, which method comprises administering to the patient an antagonist of Conrad GPCR.

24. A method of treating a patient suffering from a disease associated with reduced activity of a Conrad GPCR, which method comprises administering to the patient an agonist of Conrad GPCR

25. A method according to Paragraph 23 or 24, in which the Conrad GPCR comprises a polypeptide having the sequence shown in SEQ ID NO: 3, SEQ ID NO: 5, SEQ ID NO: 9, SEQ ID NO: 11, SEQ ID NO: 14 or SEQ ID NO: 17.

26. A method for treating and/or preventing a disease in a patient, which comprises the step of administering any one or more of the following to the patient: a polypeptide according to Paragraph 1, 4 or 5, or part thereof; a nucleic acid according to Paragraph 2, 3 or 6, or part thereof-, a vector according to Paragraph 7; a cell according to Paragraph 8; a compound according to Paragraph 16 or 17; an antibody according to Paragraph 19; a pharmaceutical composition according to Paragraph 20 and a vaccine according to Paragraph 20.

27. An agent comprising a polypeptide according to Paragraph 1, 4 or 5, or part thereof, a nucleic acid according to Paragraph 2, 3 or 6, or part thereof; a vector according to Paragraph 7; a cell according to Paragraph 8; a compound according to Paragraph 16 or 17; and/or an antibody according to Paragraph 19, said agent for use in a method of treatment or prophylaxis of disease.

28. Use of a polypeptide according to Paragraph 1, 4 or 5, or part thereof; a nucleic acid according to Paragraph 2, 3 or 6, or part thereof; a vector according to Paragraph 7; a cell according to Paragraph 8; a compound according to Paragraph 16 or 17; and an antibody according to Paragraph 19, for the preparation of a pharmaceutical composition for the treatment or prophylaxis of a disease.

29. A non-human transgenic animal, characterised in that the transgenic animal comprises an altered Conrad gene.

30. A non-human transgenic animal according to Paragraph 29, in which the alteration is selected from the group consisting of. a deletion of Conrad, a mutation in Conrad resulting in loss of function, introduction of an exogenous gene having a nucleotide sequence with targeted or random mutations into Conrad, introduction of an exogenous gene from another species into Conrad, and a combination of any of these.

31. A non-human transgenic animal having a functionally disrupted endogenous Conrad gene, in which the transgenic animal comprises in its genome and expresses a transgene encoding a heterologous Conrad protein.

32. A nucleic acid construct for functionally disrupting a Conrad gene in a host cell, the nucleic acid construct comprising: (a) a non-homologous replacement portion; (b) a first homology region located upstream of the non-homologous replacement portion, the first homology region having a nucleotide sequence with substantial identity to a first Conrad gene sequence; and (c) a second homology region located downstream of the non-homologous replacement portion, the second homology region having a nucleotide sequence with substantial identity to a second Conrad gene sequence, the second Conrad gene sequence having a location downstream of the first Conrad gene sequence in a naturally occurring endogenous Conrad gene.

33. A process for producing a Conrad GPCR polypeptide, the method comprising culturing a host cell according to Paragraph 8 under conditions in which a nucleic acid encoding a Conrad GPCR polypeptide is expressed.

34. A method of detecting the presence of a nucleic acid according to Paragraph 2, 3 or 6 in a sample, the method comprising contacting the sample with at least one nucleic acid probe which is specific for said nucleic acid and monitoring said sample for the presence of the nucleic acid.

35. A method of detecting the presence of a polypeptide according to Paragraph 1, 4 or 5 in a sample, the method comprising contacting the sample with an

antibody according to Paragraph 19 and monitoring said sample for the presence of the polypeptide.

36. A method of diagnosis of a disease or syndrome caused by or associated with increased, decreased or otherwise abnormal expression of Conrad GPCR, the method comprising the steps of. (a) detecting the level or pattern of expression of Conrad GPCR in an animal suffering or suspected to be suffering from such a disease; and (b) comparing the level or pattern of expression with that of a normal animal.

37. A diagnostic kit, according to Paragraph 22, a method according to Paragraph 23, 24, 26 or 36, an agent according to Paragraph 27 or a use according to Paragraph 28, in which the disease is selected from the group consisting of: long QT syndrome-4 with sinus bradycardia disease, mental health wellness-2 disease, psoriasis or susceptibility to psoriasis, dentin dysplasia, type II disease and neutropenia, neonatal alloimmune disease.

Various modifications and variations of the described methods and system of the invention will be apparent to those skilled in the art without departing from the scope and spirit of the invention. Although the invention has been described in connection with specific preferred embodiments, it should be understood that the invention as claimed should not be unduly limited to such specific embodiments. Indeed, various modifications of the described modes for carrying out the invention which are obvious to those skilled in molecular biology or related fields are intended to be within the scope of the claims.

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SEQ ID NO: 12

Mus Musculus Conrad cDNA

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SEQ ID NO: 16

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SEQ ID NO: 17

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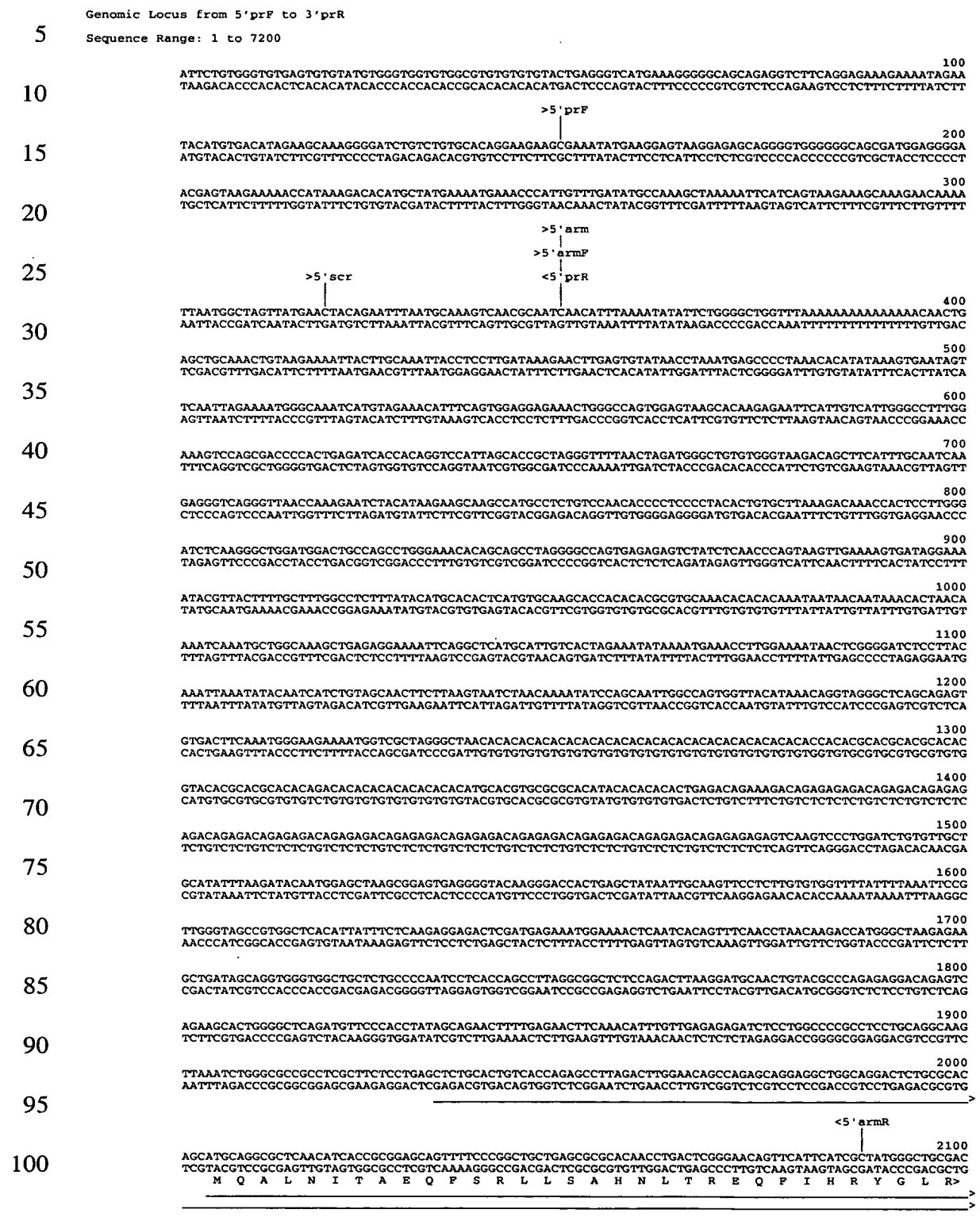
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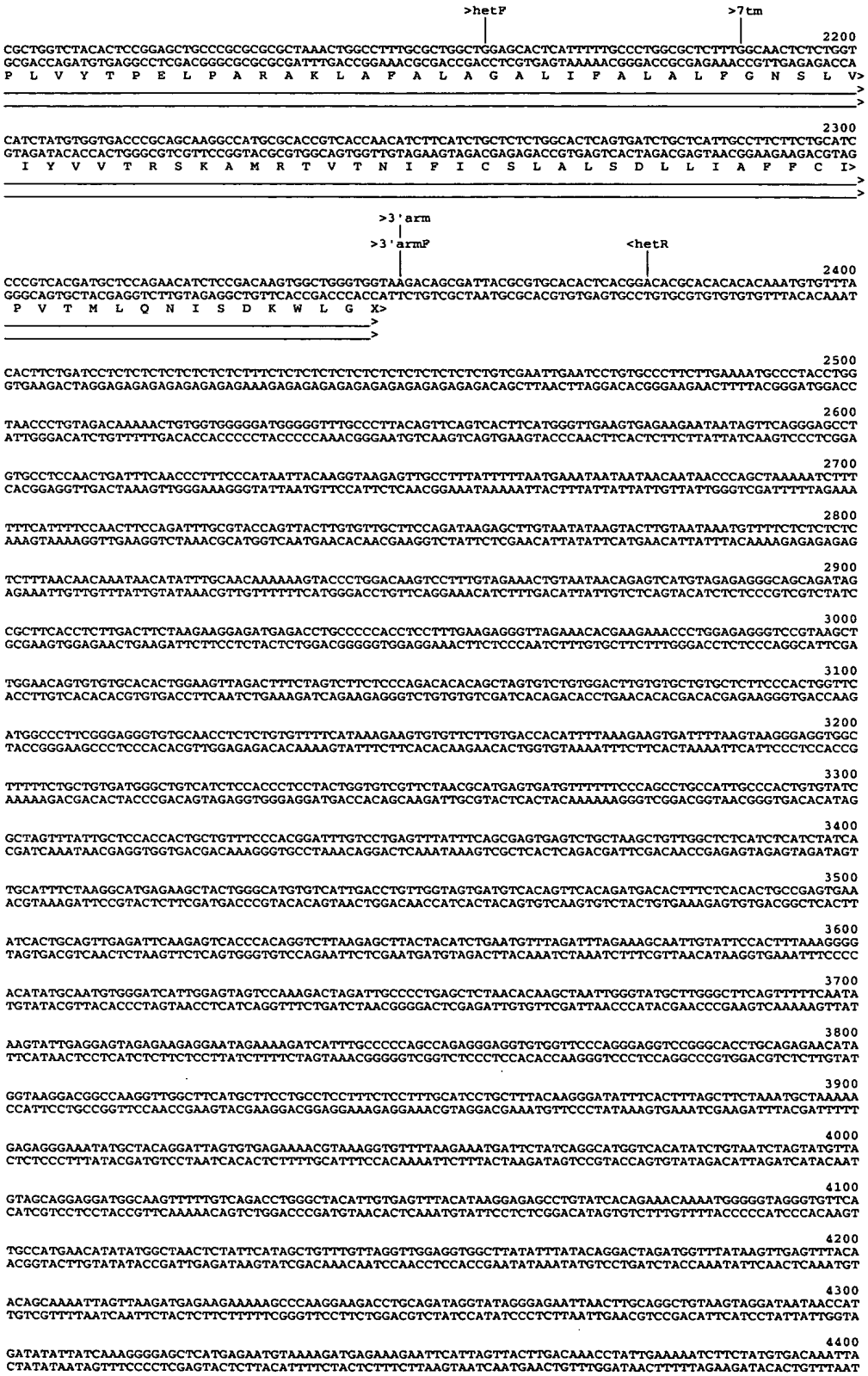
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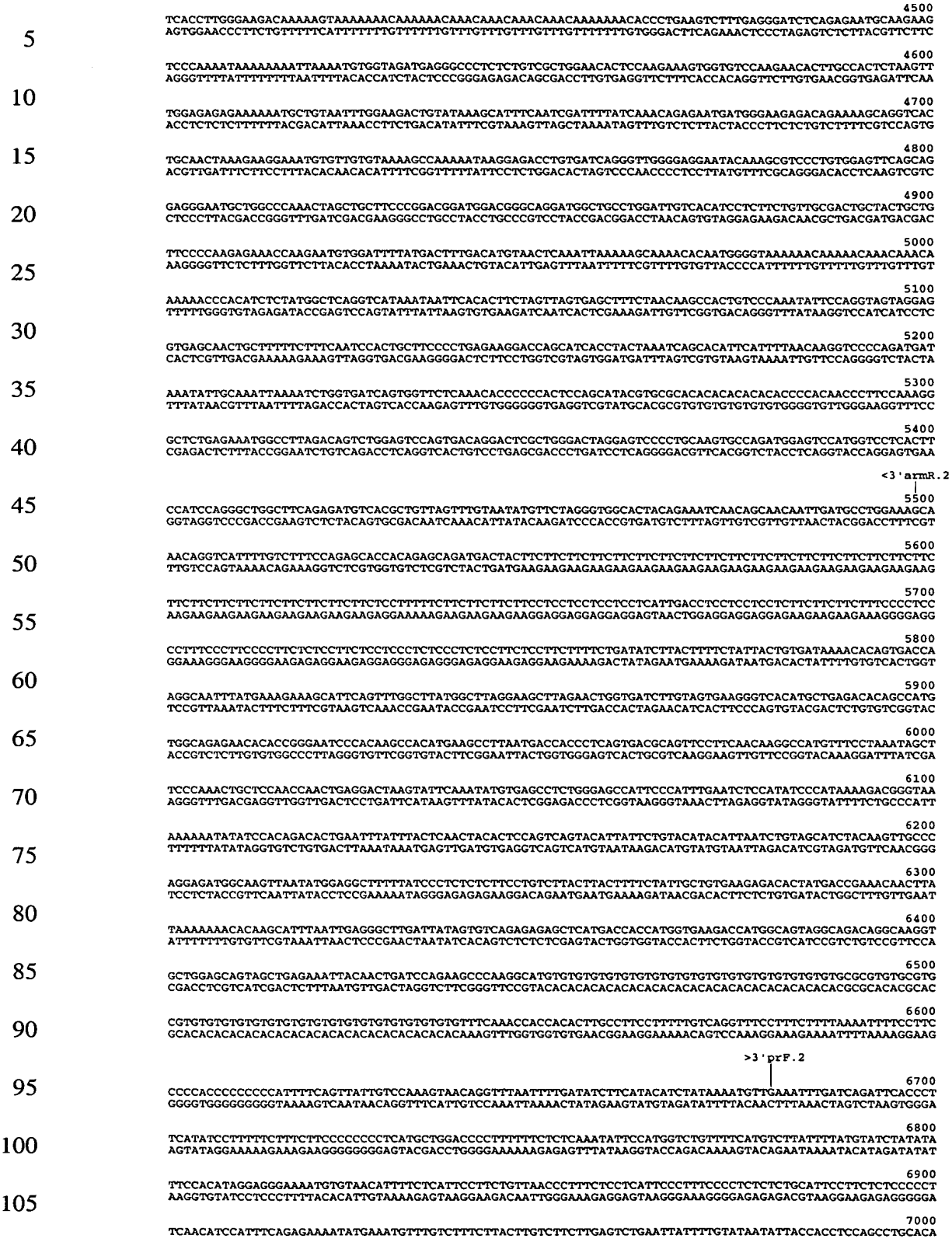
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6300
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95
6500
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100
6600
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>3'prF.2
6700
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105
6800
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6900
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7000
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      <3'prR
          |
                                                                                                  7100
TAACAATCTTATCATTCTTTATAGCTGAATAGAAGTGTGTGTGTCTGTGTGTGCATGTACATCTCTGTGTGTGTCTCTGTGCGTACATCTGTGTTTATGT
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                                                                                                  7200
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